

IN VITRO EVOLUTIONARY DYNAMICS OF *C. albicans* DURING ADAPTATION
TO FLUCONAZOLE

A Thesis

by

MIAN HUANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Chemical Engineering

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ABSTRACT

In Vitro Evolutionary Dynamics of *C. albicans* during Adaptation to Fluconazole.

(August 2012)

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Many drug-resistant mechanisms in *Candida albicans* (*C. albicans*), a clinical important fungal pathogen, have been well characterized. However, few studies investigated the emergence of drug resistance from the evolutionary perspective and little is known about the evolutionary trajectories during the adaptation to the drug. Here, we examined the evolutionary dynamics of *C. albicans* both in the presence and absence of fluconazole, a first line drug, using the visualizing evolution in real-time (VERT) method.

Evolutionary dynamics of replicate *C. albicans* populations, either in the presence or absence of fluconazole, were determined and adaptive mutants arose in the populations were systematically isolated using the VERT method. Drug susceptibility assays were performed to measure the fluconazole minimum inhibitory concentration (MIC) for the adaptive isolates from drug-exposed populations. Analysis of the evolutionary dynamics revealed that mutations arose more frequently in the presence of the drug compared to the absence of the drug and the drug-resistant mutations occurred in independent lineages, suggesting a heterogeneous nature of the populations during the adaptation. In

addition, fitness effects were evaluated for each adaptive mutant both in the presence and absence of drug and we found most of them gained significant increase in the drug resistance without a fitness cost in the absence of the drug. Interestingly, the aneuploidy and gross chromosomal rearrangements, common drug-resistant mechanisms, were not responsible for the increased resistance to fluconazole of most adaptive isolates, suggesting single-nucleotide polymorphisms (SNPs) or other stable unknown chromosomal rearrangements may contribute to the increased drug resistance.

DEDICATION

To my parents

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1. INTRODUCTION

Invasive infections caused by fungal pathogens are increasingly recognized as major challenges in public health. *C. albicans* is among the most frequent fungal species isolated from the clinical specimens and represents the most common cause of Candidiasis. The emergence of antifungal drug resistance in this organism is rapid both during the infection of a host system and laboratory adaptive evolution, further deepening the public health concern over this fungal pathogen. The drug resistance development in pathogens is a process of adaptive evolution during which genetic mutations will emerge and undergo the selection of the environmental stresses. The populations undergoing the adaptive evolution may be heterogeneous in their genotypes, depending on the population size, the rate of mutation, and the fitness effect associated with mutations. Thus, clonal interference may occur during the adaptive evolution, leading to the loss of many beneficial mutations due to the competition among multiple adaptive genotypes for expansion in populations (28, 42). Determining the evolutionary dynamics during the adaption is important for understanding the fundamental principles underlying how eukaryotic pathogens evolve resistance to antimicrobial agents, since it provides a direct observation of the population's adaptive evolution and records the

This thesis follows the style of Eukaryotic Cell.

continuous dynamic process in terms of the arising and expansion of the adaptive mutants.

The significance of the evolutionary dynamics not only lies in the useful information on adaptation provided by it, such as the frequencies with which adaptive mutants emerge and expand, but also in that it enables the systematic analysis of the adaptive events occurred in sequence during the adaption. These are important for both theoretical studies and practical applications. For example, whether the evolution of an organism follows similar adaptive mechanisms is a critical and intriguing question in theoretical study. Evolutionary dynamics enables us to analyze and determine the evolutionary trajectories (the order of occurrence of adaptive mutations) of populations during the adaptation. By comparing evolutionary trajectories determined from parallel populations we are able to gain some insights into the question. In practical applications, knowledge of the frequency and evolutionary trajectories of the drug-resistant populations and whether specific patterns of the trajectories are correlated with the level of drug resistance ultimately achieved in the populations can be used to predict the likely trajectory of a clinical infection and to develop appropriate therapeutic strategies.

In addition, although drug resistance mechanisms in *C. albicans* have been extensively studied, more new molecular bases and cellular pathway involved in the development of drug resistance remain to be characterized (136). The analysis of the evolutionary dynamics is able to facilitate the systematic isolations of the adaptive clones and thus help to discover novel drug-resistant mechanisms and relevant pathways.

The aim of this thesis was to understand how *C. albicans* evolves resistance to fluconazole, an antifungal agent, during the *in vitro* adaptive evolution, using the visualizing evolution in real-time (VERT) system. This included:

1. establishing a systemic approach to monitor the *in vitro* adaptive evolution of *C. albicans* in terms of the alteration in population structure,
2. determination of the *in vitro* evolutionary dynamics of *C. albicans* both in the absence and presence of the fluconazole,
3. identification and isolation of adaptive mutants from the evolved experimental *C. albicans* populations,
4. characterization of the drug-resistant mechanisms and evaluation of their fitness effects.

The VERT system for *C. albicans* was established to analyze the adaptation of the fungal pathogen in continuous culture under increasing concentrations of fluconazole. The evolution of the organism under same conditions except the presence of the antifungal agent served as the control. A set of isogenic strains differing only in the fluorescent markers were generated to divide the whole population into different subpopulations, which helps to visualize the occurrence of adaptive events, to measure the frequency, and to facilitate the isolation of the adaptive mutants. FACS was used to determine the proportion of different subpopulations in real time during the adaptive evolution and the collected fluorescence distribution data of the *C. albicans* populations was used to plot the evolutionary dynamics. According to the evolutionary dynamics a series of adaptive events were identified in sequence and single colonies probably responsible for the events were thus isolated. Drug resistance levels of these adaptive clones were then determined by fluconazole MIC measurements and the stability tests were performed on these clones with increased drug resistance to exclude the possibility of transient physiological response. For those adaptive clones with stable drug resistant phenotype, contour-clamped homogeneous electric field (CHEF) karyotype analysis and flow cytometry were used to examine the gross chromosome rearrangements and strain ploidy respectively. Strains displaying karyotypes that are different from the wild-type *C. albicans* underwent further microarray analysis to identify the DNA present in the

extra bands. Pairwise competition assays under similar conditions to the evolutionary environment were performed both in the presence and absence of the fluconazole to evaluate the relative fitness coefficients of all adaptive clones against either the parental strain or the previous adaptive strain.

2. BACKGROUND AND LITERATURE REVIEW

2.1 The Genus Candida

The genus *Candida* is a member of the phylum Ascomycota, the largest phylum of fungi with over 64000 species, which includes many species of industrial significance such as *S. cerevisiae* and many important human pathogens such as *Aspergillus* spp. and *Cryptococcus* spp. Species from the genus *Candida* usually colonize in human body as commensals and belong to the normal microbial flora of skin and mucosal surface, including oral cavity, gastrointestinal tract, and genitourinary tract.

Candida spp. is a group of important opportunistic pathogens. When the balance of the normal microbial flora is disrupted or the host immune system is compromised, these fungal pathogens may cause superficial infections of mucosal epithelial tissue and even life-threatening invasive infections. The superficial candidosis includes chronic atrophic stomatitis, chronic mucocutaneous candidosis and vulvovaginitis, while the severe invasive infections include bloodstream infections and infections of deep organs.

Candida spp. are the fourth most common pathogenic agents responsible for nosocomial bloodstream infection in the United States (143) and the third and fourth most common cause of urinary tract infection (10) and catheter-related bloodstream infection (84),

respectively, in Europe. In addition, they are also responsible for 13.2% of all intra-abdominal infections (104) and 4% of bloodstream infection after stem cell transplantation with a high crude mortality (42%) (88).

Although the distribution of *Candida* species in clinic isolates varies significantly across different host niches, *C. albicans* remains to be the most frequently isolated species among all *Candida* species (~50% in North America) (91). However, the role of the non-*albicans* *Candida* (NAC) in the Candidiasis is becoming more and more important, as reflected by the increase in the proportion of NAC in clinical isolates. In 1970-1990, NAC represented 10-40% of all candidemias, and increased to 35-65% in 1991-1998 (60). NAC species are more frequently isolated from cancer patients, especially those with haematological malignancies and bone marrow recipients (40-70%), and less likely from intensive care unit (ICU) and surgical patients (35-55%), children (1-35%), and HIV-infected patients (0-33%) (60). Among NAC, the most common species include *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis*, which together account for about one half of all bloodstream infections (60). *C. parapsilosis*, the second most common isolates, is mainly responsible for bloodstream infections associated with catheters, hyperalimentation, premature infants, or contaminated prosthetic devices (60). *C. tropicalis* is frequently isolated from cancer, neutropaenic and bone marrow transplant

(BMT) patients (130, 140). *C. glabrata* is also considered to be the second most common isolates responsible for systemic infections caused by *Candida* spp. according to some studies (56, 143) and has a high inherent resistance to some commonly used antifungal agents such as fluconazole. *C. krusei* has a predilection for neutropenic adult cancer patients (139) and has a high natural resistance to azoles as well. Less common *Candida* spp, such as *C. lusitaniae*, *C. guilliermondii* and other rare species such as *C. rugosa*, *C. pseudotropicalis*, *C. stellatoidea*, *C. norvegensis* and *C. famata*, have also been reported in clinics.

Compared to the *C. albicans*, some NAC species may cause more severe infections due to following two factors. First, a higher mortality rate associated with infections, especially severe systemic infections, caused by some NAC species have been reported in many studies (29, 139). Second, the high inherent resistance to antifungal agents was observed in some NAC species such as *C. krusei* and *C. glabrata*, limiting the antifungal therapy options and contributing to the increase in the failure of therapeutic treatments. Therefore, species identification is essential for the design of antifungal therapy against infections caused by *Candida* spp. and species-specific strategies should be adopted based on the causative infectious agent identified.

2.2 *Candida albicans*

2.2.1 General Characteristics of *C. albicans*

C. albicans has a heterozygous diploid genome of about 32 Mb, which is slightly larger than that of *Saccharomyces cerevisiae* (*S. cerevisiae*), and is believed to be an obligate diploid. It contains eight pairs of chromosomes which can be separated by pulse-field gel electrophoresis (75). Variability in karyotypes of the genome is frequently observed in *C. albicans* clinical isolates, mostly as a result of changes in the number of the ribosomal DNA (rDNA) repeats (98, 138) and reciprocal translocation events between multiple repeat sequences (MRS) (21, 22, 124). In addition, high frequency of genome rearrangements occur in this opportunistic pathogen through non-disjunction or other cryptic mechanisms, leading to the loss of single or multiple chromosomes or formation of isochromosomes. This has been shown to be an important mechanism for *C. albicans* to adapt to a variety of environmental stresses. For example, the loss of chromosome 5 was frequently found in strains growing on sorbose as the sole carbon source (51) and the loss of chromosome 4 and the gain of chromosome 3 have a higher incidence in strains resistant to fluconazole (90).

The genomes for two *C. albicans* strains, SC5314 and WO1, have been fully sequenced.

The sequencing of the former was completed by the Stanford Genome Technology

Center (12, 52) and the latter by the Broad Institute of MIT and Harvard. Comparative genome studies of the sequenced strains revealed that many open reading frames (ORFs) in *C. albicans* have homologues in *S. cerevisiae*, such as those related to the mating machinery (128), while others encode novel gene products, such as proteases, lipases, cell-wall proteins and genes important for host interactions. Although many *C. albicans* genes have homologues in *S. cerevisiae*, prediction of their functions based on the sequence similarity is not always accurate and sometimes may lead to wrong conclusions. A good example of genes with high sequence identity but different functions between these two fungi is *SPT3*. Mutation in *S. cerevisiae SPT3* causes a defect in filamentous growth while *spt3* mutants in *C. albicans* are hyperfilamentous (63).

Few detailed studies on the biochemical characteristics of this important opportunistic pathogen currently exist. Contradictory results were often obtained in these studies, which is not surprising due to the diverse origins of the subjects, different experimental conditions and the ability of the pathogen to switch from yeast to filamentous form. Some general metabolic characteristics of this organism were summarized here. *C. albicans* can grow on a simple defined medium with a carbon source, nitrogen source, phosphate and supplement of biotin (115). The temperature and PH range suitable for its

growth is between 20-40°C and 2-8 respectively. Growth rates of *C. albicans* on defined medium at 30°C generally ranges from 0.3 to 0.4 hr⁻¹ in continuous culture (115) and it seems no significant difference in the growth rate exists between its two distinct morphological forms.

C. albicans possesses all three major intact carbon metabolic pathways, including the Embden-Meyerhof pathway (EMP), pentose phosphate pathway (PPP), and tricarboxylic acid cycle (TAC). It can utilize a variety of carbon source, such as glucose, galactose, and maltose, and produce acid and gas. The utilization of glucose in this fungus is mainly through the EMP, since 60-80% of the glucose flux is through this pathway (9). *C. albicans* uses both glycogen and trehalose as energy store, while the glycogen was chosen to be the principle store of intracellular carbon source (123).

Studies on the respiratory system of *C. albicans* showed it is different from the classical pathway that is mediated by cytochrome c oxidase and instead it possesses an alternate oxidase similar to the alternate cyanide-insensitive pathway identified in many other plants and yeasts (46, 47, 58, 59). This alternate oxidase seems express at all stages of growth and in both filamentous forms and yeast forms (114). Cyanide and antimycin A, inhibitors of the normal cytochrome pathway, didn't affect the activity of the alternate

oxidase and so did chloramphenicol and cycloheximide (114). However, the cryptic alternate oxidase is sensitive to the presence of Salicyl-hydroxamic acid (114). All of these evidences confirmed the existence of alternate respiratory pathway in *C. albicans*.

2.2.2 Virulence Factors of *C. albicans*

Many factors have been found to be associated with the virulence in the *C. albicans* through extensive *in vivo* studies of the animal models. They include host recognition biomolecules, morphogenesis, and enzymes related to the host invasion. These factors play key roles in the pathogenicity of this organism, but it is also important to notice that these factors may not only be restricted to the pathogenic states. Thus, prediction of a pathogenic strain cannot only be made based on the existence of these virulence factors.

Adhesins, a large group of biomolecules promoting the adherence of pathogens to their host or host-cell ligands, are involved in the host recognition process. Five genes which have been assigned an adhesin function in *C. albicans* are discussed here, including *ALS1*, *ALAI*, *HWPI*, *INT1*, and *MNT1*. These genes have already been isolated and their functions were briefly described in table 1. *ALS1* of *C. albicans* has a *S. cerevisiae* homologue which encodes the α -agglutinin protein responsible for cell-cell interaction during the mating process and is a member of the agglutinin-like sequence (ALS) family.

ALAI encodes another protein belonging to the same family as *ALSI* and both of them seem to have an adhesin function (37, 40, 41). *HWPI* encodes a mannoprotein with its N-terminal domain thought to be exposed to the surface and the C-terminus covalently integrated with cell wall β -glucan (120). Disruption of the gene led to a reduced virulence in a hematogenously disseminated murine model (119). Consistently, another mutant of the gene caused a decline in the mortality and the rate of reproduction in the kidneys of infected mice and did less damage to the endothelial cells as well (127).

INT1 has been identified as a putative integrin gene in *C. albicans*(38). Genes from the integrin family of mammalian cells are required for binding to the extracellular matrix ligands. Although *INT1* of *C. albicans* has a low identity compared with the sequences of mammalian integrin genes, several evidence confirmed its role in adherence of *C. albicans* and formation of its filamentous form (39), including the binding of the *C. albicans* yeast cells to antibody that is against the divalent cation-binding domain and formation of elongated cells due to the heterologous expression of the gene in *S. cerevisiae*. In addition, the deletion of the *INT1* reduced the virulence and adherence to epithelial cells and caused a deficiency in filamentous growth on milk-tween and Spider agar. *MNT1* encodes a type II membrane protein which is required for both O- and N-mannosylation in fungi. Deletion of the gene will decrease the ability of *in vitro* adherence and cause the mutants loss of the virulence (14).

Table 1. Adhesins of *Candida albicans* and *Candida glabrata*

Adhesin	Ligand	Mutant phenotype ^b		Other	Refs
		Adherence <i>in vitro</i>	Virulence ^c		
Ais1p	HBEC, endo ^a	Reduced	Reduced	Agglutinin-like sequence proteins	22
Aia1p (Ais5p)	FN	ND	ND	Agglutinin-like sequence proteins	20,21
Hwp1p	HBEC	Reduced	Reduced	Stabilized adherence, TGase substrate	25
Int1p	Epithelial	Reduced	Reduced	The 'integrin'-like protein	28
Mnt1p	HBEC	Reduced	Reduced	Type I mannosyl transferase	31

^aAbbreviations: endo, human umbilical vein endothelial cells; FN, fibronectin; HBEC, human buccal epithelial cells; ND, not determined; TGase, transglutaminase.

^bCompared with parental cells, heterozygote or reconstituted strains; *ALS1* and *ALS5* (*ALA1*), heterologous expression in *Saccharomyces cerevisiae*.

^cHematogenously disseminated murine model.

(taken from(Richard and William, 2001)) (15)

Morphogenesis is another important virulence factor, representing the ability of cells to switch from a yeast form to a filamentous form. The filamentous form is thought to be related to the virulence and pathogenicity of *C. albicans*. However, no determinative evidence was obtained yet. A more rational perspective on the relationship between the morphology and the host invasion may stress the importance of the flexibility of the morphological change instead of the advantages of a specific morphological form in the disease development. Morphogenesis pathways of *C. albicans* were extensively studied and two of them have been characterized in details (13, 65, 68). Components of one pathway are homologous to those of *STE12* mating and pseudohyphae pathway of *S. cerevisiae*, including *CPH1*, *CST20*, *HST7*, *STE7*, and *CEK1*. *CPH1* is the *C. albicans* homologue of *STE12* and others mentioned above are homologous to components of mitogen-activated protein kinase (MAPK) pathway, which regulates the phosphorylation

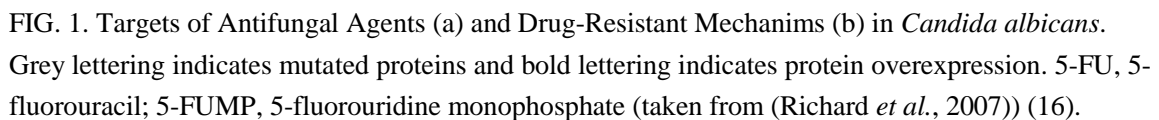
of Cph1p. In addition, Cpp1p, a protein phosphatase, is another regulatory component which regulates Cek1p in the pathway (27). Another pathway consists of Egf1p, a transcription factor, and components such as homologues of Ras proteins, adenylyl cyclase and protein kinase A (*TPK2*) (13, 65, 68, 122). This pathway is independent to the first one and mutants with one important component, *RAS1*, disrupted failed to start filamentous growth in response to serum but did form pseudohyphae (34).

Some degradative enzymes are potential virulence factors as well, including members of secreted aspartyl proteinases (SAP) family and phospholipases (PL) family. Plb1p, a typical member of PL family, encodes a glycoprotein with both hydrolase and lysophospholipase-transacylase activity. Deletion of the gene caused a reduced virulence in a murine model of candidiasis. In addition, a drop in the *in vitro* production and the activity of phospholipase has been detected at the tips of hypha during the host invasion when the gene is disrupted (43). In *C. albicans* SAP family consists of at least nine members (80). Depending on different model system, different members may be required for the host invasion of the pathogen. *SAP1-6* were correlated to the virulence in guinea pig and murine models (100), while *SAP1-3* were required for the invasion in cultured human oral tissue and lesions (105).

Phenotypic switching was a frequently observed phenomenon in *C. albicans* strains. It is usually accompanied by changes in colony morphology (such as shape, color, and surface structure) as well as differential expression of phenotype-specific genes (81, 116, 117). The plasticity of the organisms contributed by the phenotypic switching may be responsible for its ability to colonize in multiple anatomically distinct host niches as well as to adapt to a variety of environmental stresses imposed by the hosts, microbial competitors, and antifungal treatments. Thus, the phenotypic switching is a promising candidate of the virulence factors.

2.2.3 Antifungal Drugs and Drug Resistance Mechanisms in *C. albicans*

Currently, there are four major classes of antifungal agents used to treat infections caused by *C. albicans*, including the fluorinated pyrimidine analogue, polyenes, azoles and echinocandins. Each class has its own target and acts in different working mechanism, which are summarized in figure 1. Among the existing antifungal drugs, azoles are most commonly used, due to their low toxicity and their oral availability. However, the fact that they are fungistatic rather than fungicidal provides a clear opportunity for the evolution of drug resistance during long periods of prophylactic treatment (93).



C. albicans may reduce the amount of the drug that penetrates its cytoplasm membrane and reaches to the target either by changing the membrane components (71) or

expressing more multidrug transporters (73, 129). The change in the membrane components may alter the membrane barrier functions and thus block the influx of the antifungal agents into the cytoplasm, resulting in the reduction of the intracellular concentration of the drugs. On the other hand, more multidrug transporters were expressed in some mutants, leading to a more active efflux of intracellular drugs, and thus achieve the similar result. Multidrug transporters associated with drug resistance in *C. albicans* are the products encoded by *CDR1*, *CDR2*, and *MDR1*. The two *CDR* genes encode two ATP-binding cassette (ABC) transporters while the *MDR1* gene encodes a major facilitator and their upregulation were observed in many clinical azole-resistant isolates (101, 102, 141, 142).

C. albicans may alter the drug targets to minimize the drug effect on the cells by changing its amounts and structures. Either expressing more drug target or reducing the target's affinity to the drug can confer drug resistance on the cells. The major target for azoles in *C. albicans* is a cytochrome P450, encoded by *ERG11*, which catalyzes the demethylation of the sterol in its 14 α position. Drug-resistant mutants can increase the amount of the Erg11p expressed by either the duplication of the gene or upregulation of the gene's transcription (78, 136). Many point mutations in *ERG11* gene were detected in the drug-resistance clinical isolates of *C. albicans* (72, 135) and some, such as G464S

(55) and R467K (61) ,were shown to reduce the affinity of the gene product to antifungal agents by altering the heam-binding domain of the drug target.

High level of heterozygosity was found in *C. albicans* (94, 125, 132, 133) as well. The LOH in this fungus, either by mitotic recombination or chromosome rearrangement, is shown to be correlated with the drug resistance (23, 36, 135). It not only occurs in the open reading frames (ORF), but also in promoter regions. Therefore, the LOH may affect functions of proteins by altering their structures and can also change the expression patterns of genes in the transcriptional level. A good example showing the transcriptional effect of the LOH is the allelic variation in the promoter region of *SAP2*, a pathogenicity-related gene of *C. albicans*, which leads to the differential regulation of the two *SAP2* alleles (121). The LOHs at other two genetic loci, *ERG11* and *TAC1*, are of particular interest since they were correlated with the acquisition of drug resistance. In addition to LOH, aneuploidy is also shown to be associated with the emergence of drug resistance in clinical isolates and laboratory evolved strains (109, 111, 112). A study monitored the dynamics of gross chromosomal rearrangements in *C. albicans* populations during the *in vitro* evolution both in the presence and absence of fluconazole (112). In this study, multiple aneuploidies were detected in populations evolved with the drug while not found in the non drug-exposed populations and among the multiple

aneuploidies detected, formation of isochromosome 5L (i(5L)) was correlated to the acquisition of drug resistance. Interestingly, they further observed that the early appearance of i(5L) was only detected in populations which acquired high level of drug resistance in the end of their evolutions.

Furthermore, alterations in the fungal metabolism and formation of the dense biofilm may contribute to the increased antifungal resistance as well. Clinical drug-resistant strains with mutations in the *ERG3* gene were isolated and their drug resistance was correlated to the *erg3* mutations. Mutations in *ERG3* will produce inactive sterol $\Delta 5, 6$ desaturases and thus prevent the formation of toxic metabolite when normal sterol synthesis pathway was blocked (54, 87), reducing the cytotoxicity of some antifungal agents. Studies also showed that *C. albicans* cells in the biofilm are more resistant to drugs such as azoles and amphotericin B compared to their planktonic form (17).

2.2.4 Mating in *C. albicans*

C. albicans was long thought to be an asexual fungus which exists only as an obligate diploid. However, evidence suggesting the existence of mating in this fungus was reported in many studies. An important discovery related to the mating in *C. albicans* was made in 1999 by Hull CM and Johnson AD (49). They identified a mating-type like

(*MTL*) locus in *C. albicans* and genes residing at the locus include homologues of many key regulators of mating in *S. cerevisiae*, such as *MTLa1*, *MTLa1*, and *MTLa2*. In addition, a novel transcriptional regulator *MTLa2* and three pairs of open-reading frames with unknown roles in mating are present in the *MTL* locus of *C. albicans* as well, which are missing in the mating-type (*MAT*) locus of *S. cerevisiae*. Convincing evidence on the mating of *C. albicans* came from two independent reports which observed the mating between the manipulated strains in the laboratory (50, 76). Both research groups constructed homozygous *a* and *α* strains with different auxotrophic markers using a heterozygous *a/α* parental strain, dominant in natural population, and selected the prototrophic mating products. They obtained the expected prototrophic strain after mixing the two auxotrophic homozygous parental strains and the mating products are found to be mononuclear and tetraploidy. Later, the mating process of the *C. albicans* was further studied and described in more details (7, 69) and figure 2 briefly summarizes current understandings of the process in *C. albicans*.

Incorporation of white-opaque switching, a phenotypic switching discovered by Slutsky et al (117), in the mating is a major feature of *C. albicans* that is different from *S. cerevisiae*. In this phenotypic switching, cells switch from the white-phase form, appearing round and forming dome-shaped colonies on plates, to opaque-phase form,

appearing elongated and forming flatter and darker colonies on plates. In addition to the difference in the cell morphology, the rate of switching between white and opaque phase varied in different direction and transcriptional profiles of cells in different phases are different as well. In the nature environment, *C. albicans* cells have a predilection for white phase as the result of a much higher frequency of switching in the opaque-to-white direction (95) and there are about 400 genes in total that are differentially regulated between the two phases (62, 126). Connections between the mating and the phenotypic switching were found by Miller and Johnson (79), including the regulation of the phenotypic switching by genes present in *MTL* locus and a much higher efficiency of mating in cells of opaque phase versus white-phase cells. However, the exact role of white-opaque switching in the mating of *C. albicans* is not very clear and one possibility is that its incorporation is to restrict the occurrence of mating to certain host niche with appropriate environment.

Many components of the mating pathway in *C. albicans* have been identified, including a pheromone, some mating regulators, and proteins involved in the mating signal transduction cascade. The mating pheromone produced only by α cells has been found in many laboratories (8, 62, 70) and its precursor is encoded by *MFa*. In order to obtain the mature mating pheromone, the precursor needs to be further processed by Kex2p.

As mentioned above, mating regulators are also important components of the mating pathway. Those identified in *C. albicans* include four transcriptional factors. Most of them have similar functions to their counterpart in *S. cerevisiae*, while some major differences between the two organisms in mating regulators are worth notice (126). Compared to *S. cerevisiae*, *C. albicans* has a novel positive transcriptional regulator Mtl2p, an activator of a-specific genes, while the function of Mtl2p as a repressor to regulate the expression of a-specific genes in *S. cerevisiae* is absent from the fungal pathogen. In *S. cerevisiae* MAPK pathway plays a key role in transducing the extracellular mating signals into transcriptional responses in the nucleus (32, 65, 107). This signaling transduction pathway is conserved in *C. albicans*. Many components of the signaling pathway in *C. albicans* are homologous to their counterparts in *S. cerevisiae* and these signaling proteins are required for the mating in *C. albicans* as well (18, 74). An obvious difference in the signaling cascade between the two organisms is the absence of the homologue of *S. cerevisiae* *STE5* in *C. albicans*, which encodes a scaffold protein holding the signaling components together to prevent the interference from other signal pathways using the same signaling components. Furthermore, evidence suggests other signal transduction pathways may also be involved in the regulation of mating in *C. albicans*, such as nutritional signals (8, 18, 19, 69).

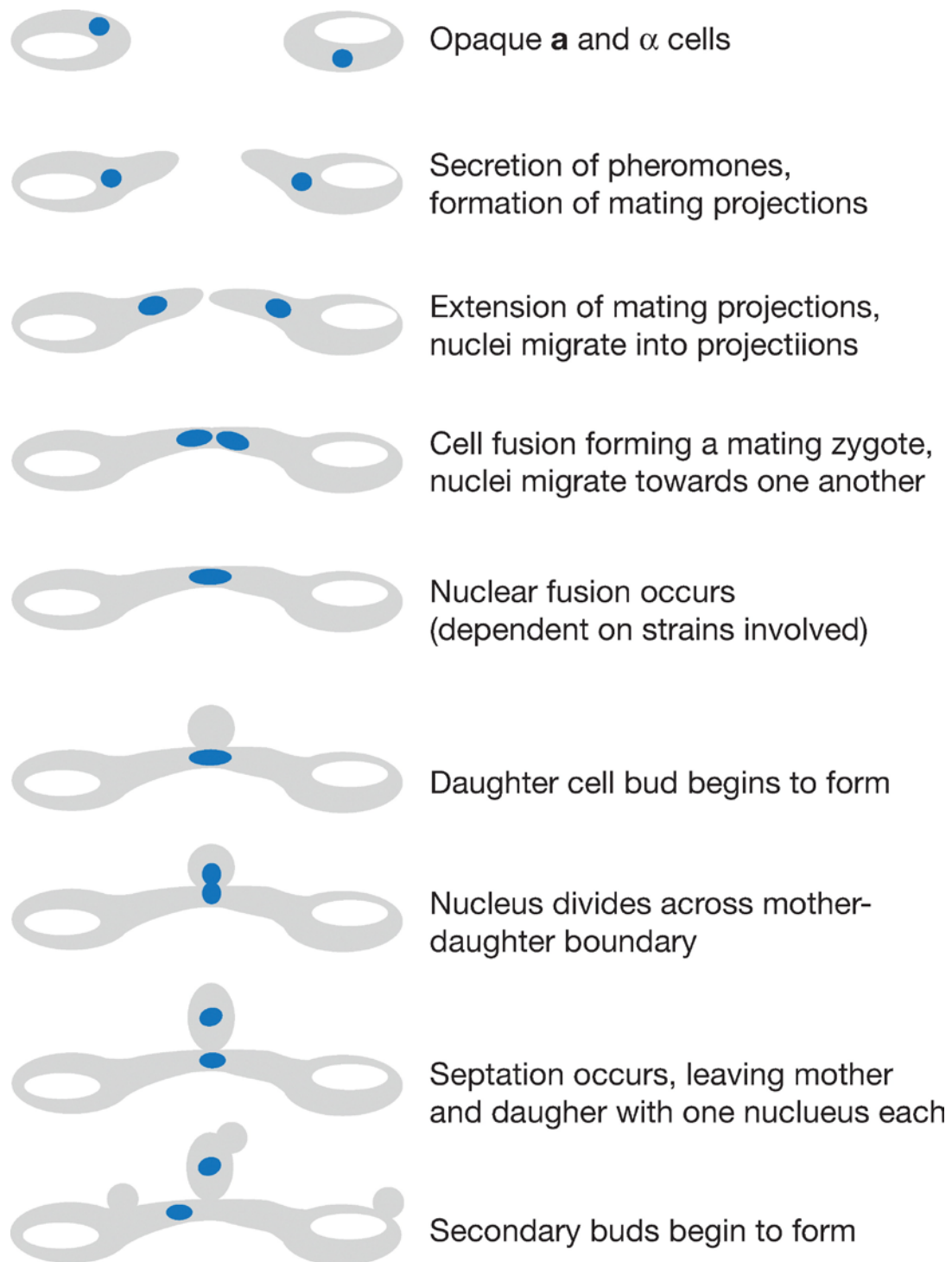


FIG. 2. Steps in the Mating of *Candida albicans*. Nuclei are depicted in blue and vacuoles in white. The parental mating cells are diploid, and the mating products are mononuclear and tetraploid. The vacuole is not depicted in the daughter cell (taken from (Bennett and Johnson, 2005))(6).

2.3 Developments in the Genetic Manipulation of *C. albicans*

Genetic manipulation of *C. albicans* is still a challenging task for researchers after decades of extensive exploration. Many factors contribute to the difficulties in manipulating the genome of *C. albicans* and most of them are related to the unique features of this fungal pathogen, including the lack of a defined sexual cycle (86), its diploid DNA content (5, 52), lack of plasmids that can be transformed with high efficiency, different usage for CUG codon (103), and a few selective markers available.

In the genetic manipulation of *C. albicans*, the limited number of selective markers can be divided into three types. The first one is the nutritional markers and manipulation using this type requires a special parental strain which is engineered to be auxotrophic for growth on the media without the supplement of nutrients specified by the selective markers. The first nutritional marker that is widely used in *C. albicans* is *URA3* marker. It was Fonzi and Irwin who made the first reliable strain, CAI4, for introduction of mutations using this marker (35). Although the use of *URA3* marker offered many advantages and many methods were developed based on this marker to manipulate the genome of *C. albicans*, its drawbacks gradually appeared during the extensive applications. A major problem is that the expression of the *URA3* at ectopic loci can affect the virulence, adherence, and hyphal morphogenesis of *C. albicans* (11, 20, 64).

To overcome the drawback, the use of *LEU2*, *HIS1*, *ARG4* as nutritional markers has been evaluated and corresponding auxotrophic strains were constructed (85). The second type is the drug resistance markers. Compared to the nutritional markers, using this type of markers in genetic manipulations doesn't need to create auxotrophic strains or worry about the influence of the nutritional status on the genes of interest. To my knowledge, *IMH3* marker, conferring resistance to mycophenolic acid (MPA), might be the first drug resistance markers used in the genetic manipulations of *C. albicans* (57, 141). Another two useful drug resistance markers are *SAT1* and *CaNAT1*, encoding streptothricin acetyltransferase and nourseothricin acetyltransferase respectively (92, 113). Both these two dominant markers confer the resistance to nourseothricin in *C. albicans*. Recently, fluorescent markers are used in the disruption of target genes in *C. albicans*, such as green fluorescence protein (GFP) marker, and codon-optimized luciferase markers from firefly and Renilla (30, 82, 118).

To manipulate the genes in *C. albicans*, many strategies and gene cassettes, assisting the disruption of genes of interest, have been developed (briefly introduced in figure 3).

Advantages and drawbacks of some popular methods and cassettes were discussed here.

These methods and cassettes can be classified into two major classes according to whether their target genes are essential or not. To disrupt a non-essential gene, methods

such as URA Blaster method, URA Flipper method, method using UAU1 cassette, and their derivatives are widely used. URA Blaster method makes use of the auxotrophic strain CAI4 and *URA3* nutritional marker to disrupt the genes of interest. The nutritional marker can be recycled in this method for next round of disruption by looping out the marker from the genome. In addition, the excision of the *URA3* marker can be counter-selected on 5-Fluoroorotic acid (5-FOA). However, the auxotrophic strain used in this method, CAI4, was shown to have defects in expressing certain proteins compared to the wild-type strain SC5314 (11) and the method does leave one copy of the *hisG* sequence in the target site which might influence the expression of the adjacent genes.

Furthermore, the counter selection of the *URA3* marker requires a potential mutagenic agent, 5-FOA (131). Compared to the URA Blaster method, URA Flipper method (83) avoids the use of the mutagenic agent to counter select for its nutritional marker *URA3*, however the recycling of the marker requires nutritional changes from normal growth media to Yeast Carbon Base and Bovine Serum Albumin, pH4.0 (YCB–BSA) medium. The URA Flipper method also leaves behind a FRT sequence in the genome but much shorter compared to the *hisG* sequence left by the URA Blaster method. Both the two aforementioned methods need several cloning steps to disrupt a gene of interest in *C. albicans*, but Enloe *et al.* developed a novel strategy based on the UAU1 cassettes they made, which is able to disrupt both two alleles of the gene of interest with just one

transformation step (33). This gene cassette contains a *URA3* marker that is disrupted by *ARG4* marker and when the *ARG4* marker is looped out due to the spontaneous homologous recombination, the *URA3* marker regained its functions. Thus, simultaneous selection of both the two markers could identify the double-deleted transformants. Use of this method doesn't require both the nutritional change and the mutagenic agent, but the *URA3* marker will be left in the locus of the disrupted genes, which, as mentioned above, may bring some concerns.

Compared to the disruption of a non-essential gene, disruption of an essential gene requires a more complicated system and usually more cloning steps to make sure the expression of the essential gene only under permissive conditions. Two popular approaches to make the conditional mutants are the gene replacement and conditional expression (GRACE) method (96) and a method (89) based on the "Tet-On" system constructed by Gossen *et al* (45). In the GRACE method, one copy of an essential gene is disrupted while the other is engineered to be controlled by the tetracycline (figure 3-line 10). The tetracycline (Tet) regulation system used in GRACE method turns off the expression of the target gene in the presence of the Tet. In contrast, both two native copies of essential genes are deleted in the method using the "Tet-On" system. The requirement of the essential gene in this approach is satisfied by integration of a cassette

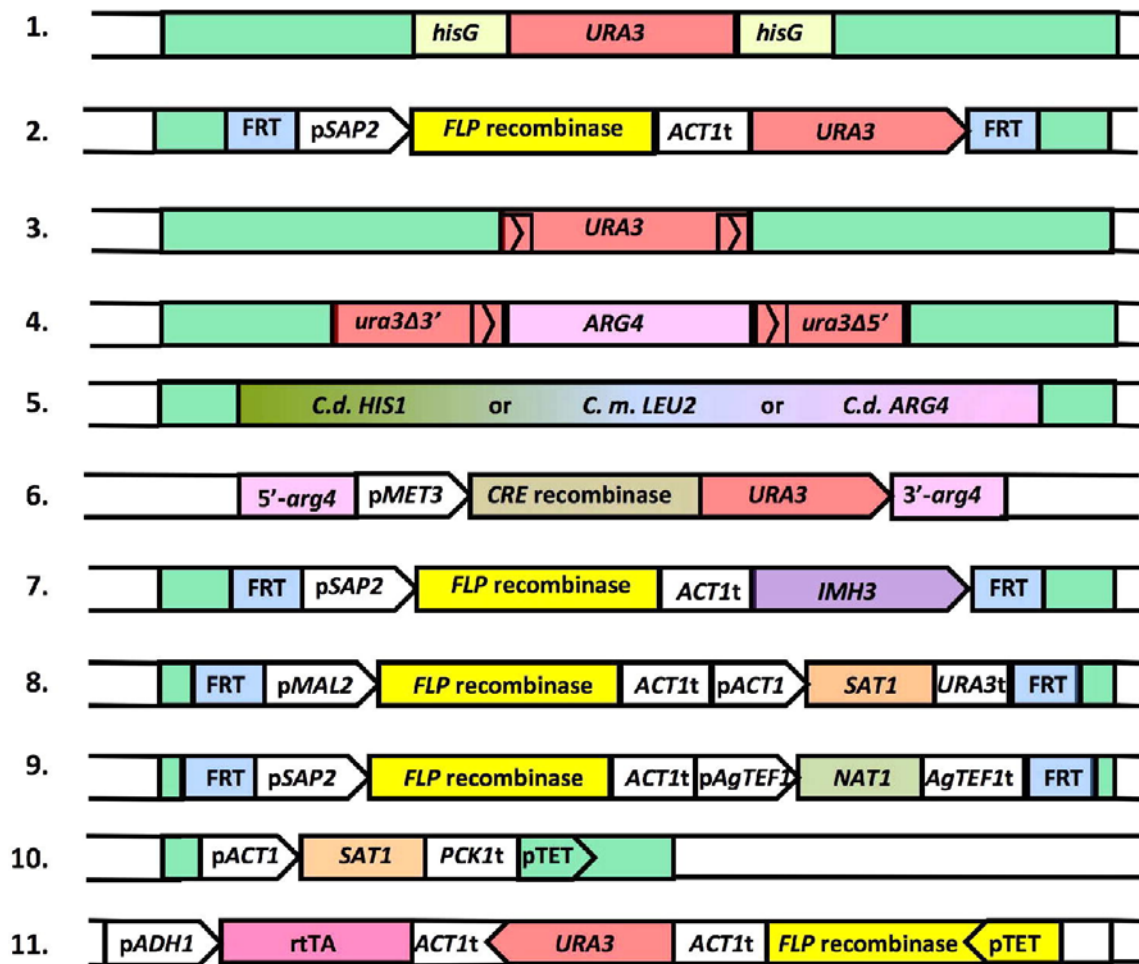


FIG. 3. Genetic Manipulation Tools of *Candida albicans*. The figure shows the various constructs used for gene manipulation in *C. albicans*. The gene of interest and/or flanking sequences are shown in green. All sequences coming from different species have been codon optimized to be used in *C. albicans*. Line 1. URA blaster (Fonzi and Irwin, 1993), 2. URA flipper cassette. p; promoter region of given gene, t; termination sequence of given gene. (Morschhäuser *et al.*, 1999), 3. PCR amplifiable URA3 disruption cassette (Wilson *et al.*, 2000), 4. UAU1 cassette (Enloe *et al.*, 2000), 5. PCR amplifiable marker cassettes from non-albicans species. C. d.; *Candida dubliniensis*, C. m.; *Candida maltosa*. (Noble and Johnson, 2005), 6. Cre-loxP system (Dennison *et al.*, 2005), note that when this construct integrates, the *arg4* sequences will be flanked by loxP sites, 7. “MPAR flipper” cassette (Wirsching *et al.*, 2000), 8. SAT1 flipper cassette (Reuss *et al.*, 2004), 9. CaNAT1-FLP cassette. Ag; *Ashbya gossypii*. (Shen *et al.*, 2005), 10. “Tet-Off” system (Roemer *et al.*, 2003), and 11. “Tet-On” system (Park and Morschhäuser, 2005) (taken from (Samaranayake and Hanes, 2011)) (99).

containing a copy of the gene and MPA resistance marker flanked by FRT sites into the *ACT1* locus. The “Tet-On” system constructed in another cassette is introduced into an *ADHI* locus and will loop out the cassette carrying the target gene by expressing the *FLP* recombinase gene when Tet or its derivative is present.

Many strategies and genetic systems have been developed for *C. albicans* in the past two decades, which benefits the research community of this fungal pathogen. However, compared to other model microbial systems, more genetic tools and selective markers are required for *C. albicans*.

2.4 Evolutionary Studies

2.4.1 Systems Used to Study the Adaptive Evolution of *C. albicans*

Evolutionary studies on the emergence of drug resistance in *C. albicans* are categorized into two major types based on the systems used to evolve the parental strains. In the first type, strains are isolated either from the patients receiving antifungal therapy (111, 134) or animal models established to study the pathogenesis of different types of Candidiasis, especially the murine models (3, 4). *In vivo* systems are used in this type of study, which provides ideal tools to study how the drug resistance emerges and expands in the whole population in a more realistic situation. However, variables such as population

size inside the host, genetic compositions of the founding pathogen population, and host physiological states are difficult to control *in vivo*. These factors are likely to alter the evolutionary outcomes and make evolution experiments based on these *in vivo* systems difficult to reproduce.

Another type of system used in evolutionary studies is the *in vitro* system where cell populations are evolved in more controllable and reproducible environments. The genotypes and population size of the starting population as well as the selection strength can all be readily controlled, allowing reproducibility of the environmental conditions for each experiment. *In vitro* systems can be further divided into two types, either the batch serial transfer (24-26) or the continuous cultures (48). In batch serial transfer experiments, the population is grown either on solid or liquid media, and a small fraction is serially transferred to fresh media containing the antifungal agent periodically. The population undergoes different growth phases during batch cultivation, as the nutrient content of the environment and the physiological state of the cell both change as a function of time. Compared to the batch serial transfer, continuous culture systems provide a more constant environment, which helps to keep cells in physiological steady-state. In addition, continuous culture systems greatly reduce the bottleneck effect which causes a significant loss of rare beneficial mutations and favors the selection of

deleterious mutations. Although *in vitro* systems may not exactly mimic the realistic environments, antifungal resistant mechanisms developed *in vivo* have not been found to be different from those *in vitro*. Therefore, *in vitro* systems can provide important and useful information on the drug resistance development during the evolution.

2.4.2 *In vivo* Evolution of *C. albicans* in the Presence of Antifungals

Although evolutionary studies are extremely useful in understanding the drug resistance development of *C. albicans* population, only a few studies are available. In *in vivo* studies, strains either isolated from animal models treated with antifungal agents or patients receiving the antifungal therapy are usually under the investigation to track the alteration in drug susceptibility of the isolates or the change in the transcriptional profiling during the evolution. This type of studies not only benefits to the identification of pathogenesis-related genes which may serve as novel drug targets but also to the rational design of the novel dosing regimens of current drugs.

In one study people investigated the impact of different fluconazole dosing regimens, including different dose levels and dosing intervals, on the selection of the isogenic resistant strains from six different heterogeneous populations, each consisting of a predominant drug-susceptible strain and an isogenic drug-resistant one, during a short-

term *in vivo* evolution of *C. albicans* using a murine model (3). For the same total dose level, they found the growth of resistant strain was less when it was administrated at a lower but more frequent dosage compared to higher but more infrequent one. The drug exposure was further correlated with the outgrowth of the isogenic resistant cell population when it was expressed by either the range of $T > MIC$ or 24-h AUC/MIC value and these relationships were independent of the type of resistant mechanism possessed by the populations. The results were confirmed by another study from the same group which used the same murine model, but cell populations were evolved from a single drug-sensitive strain instead of a mixed-inoculum for a month (4). These studies suggest different selection strategies may have different impacts on the expansion of the drug-resistant genotypes in the population, develop different population dynamics and ultimately lead to different evolutionary outcomes. In addition, population structure prior to the evolution is another important factor. One piece of evidence came from the aforementioned short-term evolution study (3). They found if populations initially contained at least 10% of drug-resistant clone, the evolved populations would behave phenotypically as entirely drug-resistant, suggesting that the population structure plays a key role in determining the evolutionary outcome. Thus, no matter what selection strategy was adopted, the expansion of the drug-resistant phenotype in the entire

population seems cannot be prevented when the population size of the drug-resistant mutants grows big enough.

Researchers also studied the change of mRNA level of three candidate genes, including *CDR1*, *MDR1*, and *ERG16*, in a series of 17 clinical isolates from a HIV-infected patient over two years (134). They successfully correlated the increase in mRNA level of the genes with the increased drug resistance of the isolates based on the timing of the two events. What they found clearly showed multiple mechanisms can occur in the same cell line simultaneously and contribute to the final drug-resistance level.

2.4.3 *In vitro* Evolution of *C. albicans* in the Presence of Antifungals

Similar to the *in vivo* evolutionary studies little information on drug resistance development of *C. albicans* during the *in vitro* evolution is available. Among the few relevant studies Cowen *et al.* did a series of elegant work to pave the way for the investigations of the *in vitro* adaptive evolution of this fungus. They evolved 6 replicate populations, found from a single drug-susceptible clone, both in the presence and absence of fluconazole over 330 generations using the serial batch transfer method and monitored the cell populations' adaption to the inhibitory concentration of fluconazole (26). By observing the change of fluconazole MIC over time in replicate populations

Cowen *et al.* found population evolution may take different adaptive paths, indicated by the significantly different trajectories of the increase in their fluconazole MICs and different drug resistance level achieved at the end of the evolution. The highest fluconazole MIC value achieved is 128-fold larger than the lowest one among these replicate populations. These observations suggest that the acquisition of the drug resistance in these replicate populations is a random process and thus chance plays a key role in the drug resistance development process.

The seemingly random process of drug resistance development raises an interesting question: whether or not the fungal populations will converge on similar adaptive paths under the selection of an antifungal agent. Cowen *et al.* further checked the genome-wide gene expression patterns of several replicate populations evolved from their previous work (26) and found that the evolution of *C. albicans* in the presence of the fluconazole might converge on a limited number of adaptive paths at the transcriptional level based on the similarity of genome-wide expression profiles between replicate populations (25). In addition, they analyzed the expression patterns of ten genes, either showing the largest changes in microarrays or involving in the drug resistance, in many drug-resistant clinical isolates and confirmed the patterns identified *in vitro* are also common to those evolved *in vivo*.

Furthermore, they measured the relative fitness of the evolved populations to a genetically marked wild-type strain both in the presence and absence of the drug (24). They found fluconazole MIC value and other growth parameters of those drug-exposed populations cannot be used to predict their fitness over the wild-type strain in the presence of the drug. Fluconazole MIC value is well-accepted as an indicator to evaluate the resistance of *C. albicans* to the drug. However, the discordance between the MIC value and the fitness results showed MIC itself may not sufficiently predict the response of *C. albicans* to antifungal treatments. Moreover, the study suggests the adaptation to the culture condition or general environment may lead to an improved fitness in the presence of drug.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Yeast Media

All media were autoclaved at 121°C. Heat sensitive substances were sterile filtered and added to the media (~56°C) afterwards. For all plates, the final concentration of agar is 2% (wt/vol).

Rich medium:

Rich medium (YPAD) was prepared with 1xYPD and adenine added to a final concentration of 40µg/ml.

	<u>1xYPD</u>
Yeast extract	10g/l
Peptone	20g/l
Dextrose	20g/l
Adenine	40mg/l

NAT-plates: Nourseothricin (100 mg/ml, H₂O) was added to a final concentration of 400µg/ml.

Minimum medium (MM):

Minimum medium was prepared with YNB without amino acid (AA) and ammonium sulfate (AS), 0.5% (wt/vol) ammonium sulfate and either 0.2% (wt/vol) or 2% (wt/vol) glucose for different purpose.

	<u>1xMM (0.2% glucose)</u>	<u>1xMM (2% glucose)</u>
YNB w/o AA & AS	1.7g/l	1.7g/l
Ammonium sulfate	5g/l	5g/l
Dextrose	2g/l	20g/l

3.1.2 Buffers and Solutions

All the buffers and solutions (table 2) used in this study were either autoclaved or sterile filtered.

3.1.3 Chemicals and Computing Software

Chemicals used to make buffers, solutions, and culture media in this study were listed in the table 3. The table 3 also shows the computing software used in this study to perform the plasmid analysis and the flow cytometry analysis.

TABLE 2. Buffers and Solutions Used in This Work

Buffer/ solution	Chemical	Concentration
LiAc/TE	LiAc	100 mM
	TE, pH7.5	1 X
PBS	NaCl	137 mM
	KCl	2.7 mM
	Na ₂ HPO ₄	10 mM
	KH ₂ PO ₄	2 mM
PEG/TE/LiAC	LiAc	100 mM
	TE, pH7.5	1 X
	PEG (3350)	0.4 g/ml
TBE	Tris-borate	45 mM
	EDTA	1 mM
TE	Tris-Cl	100 mM
	EDTA	10 mM
50/50 TE	Tris-Cl, pH8.0	50 mM
	EDTA	50 mM

TABLE 3. Chemicals and Computing Software Used in This Study

Manufacturer	Location	Chemicals
ACROS	USA	D(+)-glucose, Adenine
Becton Dickinson	USA	Peptone, agar, Yeast Nitrogen Base
EMD Chemicals	Germany	Yeast extract, $(\text{NH}_4)_2\text{SO}_4$, NaCl
Electron Microscopy Science	USA	PEG 3350
Fisher Scientific	USA	EDTA
Jena Bioscience	Germany	Nourseothricin
LKT laboratories	USA	Fluconazole
MP Biomedicals	USA	LiAc
Promega	USA	Tris-Cl
VWR	USA	KaCl, KH_2PO_4 , Na_2HPO_4

Software	Manufacturer	Location	Resource
MacVector v11	MacVector, Inc	USA	CD, license
CellQuest Pro	BD Biosciences	USA	CD, license

3.1.4 Organisms

TABLE 4. Fungal Strains Used in This Study

Strain	Genotype	Reference
SC5314	<i>C. albicans</i> wild type strain	William Fonzi via Magee lab
KKCa-14	Isogenic to SC5314; <i>ENO1-RFP-NATI</i>	This work
KKCa-17	Isogenic to SC5314; <i>ENO1-GFP-NATI</i>	This work
KKCa-20	Isogenic to SC5314; <i>ENO1-YFP-NATI</i>	This work

3.2 Methods

3.2.1 Cultivation and Storage of *C. albicans*

C. albicans strains were cultured at 30°C in liquid or solid YNB medium supplemented with 0.5% (wt/vol) ammonium sulfate and 2% (wt/vol) D-glucose unless otherwise specified. For selection of nourseothricin resistant transformants, YPAD agar plates supplemented with 400µg/ml nourseothricin were used. For short term storage cells were kept on YNB plates at room temperature in the dark. For long term storage the cells were suspended in ~17% sterile glycerol and then stored in cryogenic tubes at -80°C.

3.2.2 Transformation of *C. albicans* by Heat Shock

For heat shock transformation, we used a modified lithium acetate method (44). Cells were first grown overnight at 30°C in YPAD broth to $1-2 \times 10^8$ cells/ml and then diluted to $2-5 \times 10^6$ cells/ml in fresh YPAD medium. The diluted cells were incubated at 30°C to reach a density of 1×10^8 cells/ml. The cells were harvested by centrifugation, washed twice with 10 ml sterile water, once with 10 ml LiAc/TE (table 2), resuspended in LiAc/TE to give $\sim 2 \times 10^9$ cells/ml, and incubated at 30°C for 15 min without agitation. Cells were then distributed into 50 µl aliquots and following solutions were added in sequence: DNA fragments to transform, 50 µg heat-denatured salmon sperm DNA, 300 µl PEG/TE/LiAC (table 2). After mixing thoroughly the cells were incubated at 30°C for 30 min, heat-shocked at 42°C for 20 min and pelleted. The supernatant was completely removed and cell pellet was resuspended in 1 ml YPAD broth and plated on selective plates after 3 hours of incubation at 30°C.

3.2.3 Construction of Fluorescent *C. albicans* Strains

All strains used in this work are isogenic derivatives of the *C. albicans* strain SC5314 (table 4). Three different fluorescent cassettes, each containing a NAT1 marker and a fluorescent protein (either red fluorescent protein [RFP], green fluorescent protein [GFP], or yellow fluorescent protein [YFP]), were amplified by PCR from plasmids pMG2261,

pMG2120, and pMG2263 (table 5), respectively, using two different sets of primers (table 6). The primers for PCR amplifying GFP and YFP cassettes were Ca_Y/GFP_Fp and Ca_Y/GFP_Rp. The primers for amplifying the RFP cassette were Ca_RFP_Fp and Ca_RFP_Rp. The resulting PCR products were then transformed into strain SC5314 to form a translational fusion of fluorescent cassettes to the 3' end of the *ENO1* gene in the chromosome by homologous recombination. Transformants were selected on yeast extract-peptone-dextrose plus adenine (YPAD) agar plates with nourseothricin (400µg/ml), and the fluorescence of each strain was confirmed by microscopy.

TABLE 5. Plasmids Used in This Study

Name	Marker	Description	Reference
pMG2120	Amp/NAT	Carries GFP	Maryam Gerami-Nejad
pMG2261	Amp/NAT	Carries RFP	Maryam Gerami-Nejad
pMG2263	Amp/NAT	Carries YFP	Maryam Gerami-Nejad

TABLE 6. Oligonucleotides Used in This Study

Name	Sequence	Description
Ca_Y/GFP_Fp	5'-GAGAATCGAAGAAGAATT	Forward primer to amplify the
	AGGTTCTGAAGCTATCTACGC	YFP or GFP cassette from
	TGGTAAAGATTTCCAAAAGGC	pMG2263 and pMG2120
	TTCTCAATTGGGTGGTGGTTCT	respectively
	AAAGGTGAAGAATTATT-3'	
Ca_Y/GFP_Rp	5'-TTTAATTAGTTCATATATTC	Reverse primer to amplify the
	AAGATGTTTCCTATAAAAGAAA	YFP or GFP cassette from
	AAAAAAGCACCAGCTTTTTTTT	pMG2263 and pMG2120
	ATTTAATCGTAAAACGACGGC	respectively
	CAGTGAATTC-3'	
Ca_RFP_Fp	5'-GAGAATCGAAGAAGAATT	Forward primer to amplify the
	AGGTTCTGAAGCTATCTACGC	RFP cassette from pMG2261
	TGGTAAAGATTTCCAAAAGGC	
	TTCTCAATTGGGTGGTGGTGA	
	TAACACTGAAGATGTTATT-3'	
Ca_RFP_Rp	5'-TTTAATTAGTTCATATATTC	Reverse primer to amplify the
	AAGATGTTTCCTATAAAAGAAA	RFP cassette from pMG2261
	AAAAAAGCACCAGCTTTTTTTT	
	ATTTAATCGTAAAACGACGGC	
	CAGTGAATTC-3'	

3.2.4 Adaptive Evolution of *C. albicans*

Cells expressing each fluorescent protein (GFP, YFP, or RFP) were mixed together in approximately equal numbers (as confirmed by flow cytometry) at the start of parallel chemostat (30 ml each) evolution experiments in YNB supplemented with 0.5% (wt/vol) ammonium sulfate and 0.2% dextrose (the limiting nutrient) with or without fluconazole. The populations evolved at steady state at 30°C at a dilution rate (D) of $\sim 0.2 \text{ h}^{-1}$. In chemostat experiments, the generation time is determined by the dilution rate. Using a dilution rate of 0.2 h^{-1} , the generation time is calculated as $\ln(2)/0.2$, or 3.5 h/generation. Two parallel series of evolution experiments were conducted; each series contained one control population (no fluconazole) and one population exposed to stepwise increases of fluconazole, starting with $0.25 \mu\text{g/ml}$ and increasing the concentration by 4-fold every 50 to 70 generations. Samples were taken every 7 to 14 generations, and a portion (1.5 ml) of the cells was frozen in 17% glycerol for future analysis. Cell concentrations were measured using a hemocytometer, and the relative proportions of the three subpopulations (GFP, YFP, and RFP) were measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

3.2.5 Identification and Isolation of Adaptive Mutants

At the conclusion of the evolution experiment, we identified the time point samples likely to have a substantial fraction of an adaptive clone in one of the fluorescently marked subpopulations, based on the dynamics of the relative subpopulation sizes. Specifically, we identified the time point sample in which an expanding colored FP subpopulation appeared to reach its maximum proportion of the population. Population samples from the generation at which the expanding subpopulation reached a maximum proportion were plated on YNB plates. Using a Dark Reader (Clare Chemical), which can distinguish GFP-, YFP-, and RFP-expressing cells, at least seven isolates expressing the colored fluorescent protein of the expanding subpopulation were picked for fitness measurements. For all isolates from the same adaptive event, we performed pairwise competition experiments by growing each isolate in continuous culture together with the previous adaptive mutant (for the first observed adaptive event, the isolates were individually competed against the parental strain of a different FP). The experiments were carried out in the presence of the same concentration of fluconazole used during the evolution of those isolates. Of the seven or more clones tested from each observed adaptive event, the clone with the highest fitness coefficient was chosen as the adaptive clone for that adaptive event, except in cases where multiple isolates exhibited similar fitness coefficients. In those cases, an arbitrary isolate was chosen from the fittest group.

3.2.6 Pairwise Competition Experiments

Chemostat experiments in YNB with 0.2% dextrose and 0.5% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$ and in the presence or absence of fluconazole were initiated with approximately equal numbers of the two clones to be competed (each carrying a different FP marker). The pairwise competition experiments were conducted at 30°C with a dilution rate of $\sim 0.2 \text{ h}^{-1}$.

Samples were taken every 1 to 5 generations, and the relative proportions of each of the two clones were measured using flow cytometry. The data were plotted on a linear scale with $\ln[x/(1-x)]$ versus t , where x is the fraction of the sample clone and t is generations.

The relative fitness coefficient was determined from the slope of the linear region of the curve using linear regression analysis. For statistical analysis, the relative fitness coefficients between pairs of the fluorescently labeled parental strains were measured either in the absence of fluconazole or in the presence of 0.25 $\mu\text{g/ml}$ of fluconazole. A minimum of three biological replicates were performed for each pairwise competition experiment. A Student t test was used to determine whether the relative fitness coefficients measured for the adaptive mutants were statistically significant (P value cutoff, 0.05).

3.2.7 Fluconazole Susceptibility Assays

Adaptive mutants were first grown in YNB broth overnight at 30°C overnight and then transferred to RPMI 1640 agar plates. The fluconazole MIC for each adaptive mutant was determined in RPMI 1640 plates with an Etest strip according to the manufacturer's instructions (bioMérieux SA, France). 3 biological replicates were made for each adaptive isolates and the highest MIC value was reported.

3.2.8 Stability Assays for Adaptive Mutants

One of the parent strains (KKCa-14) and all isolated adaptive mutants from the DP-1 and DP-2 populations underwent stability tests in the absence of fluconazole. The strains were first plated on YNB plates supplemented with 2% dextrose, and a single colony was picked up and transferred into fresh YNB broth. Five microliters of cell culture for each strain was inoculated into 5 ml fresh YNB broth to make a 1,000-fold dilution and then was incubated at 30°C for 24 h. The transfer was repeated for two more days (for a total of ~30 generations). The optical density of the cell culture was measured at 600 nm before each transfer. Fluconazole susceptibility assays were performed for each strain to test the stability of the drug resistance after long-term incubation in serial batch transfer in the absence of the drug.

3.2.9 CHEF Karyotype Analysis

Preparation of cells for contour-clamped homogeneous electric field (CHEF) karyotype analysis was performed as described previously (108). Whole-chromosome separation was performed on a Bio-Rad CHEF-DR III with the following program: 60- to 120-s switch, 6 V/cm, 120° angle for 36 h, followed by a 120- to 300-s switch, 4.5 V/cm, 120° angle for 12 h. SfiI digestion of CHEF gel plugs was performed using the following procedure. A portion of whole-chromosome CHEF plugs were washed twice for 10 min each time in 1 ml of 10 mM Tris (pH 8.0), 1 mM EDTA (TE buffer) and then incubated in 1 ml of TE buffer containing 0.1 mM phenylmethylsulfonyl fluoride for 10 min. The plugs were washed twice as before and incubated in 500 µl of SfiI restriction enzyme buffer containing bovine serum albumin (BSA) at 100 µg/ml for 15 min, and 40 units of SfiI enzyme was added. The SfiI-treated plugs were then incubated overnight at 50°C, washed in 100 mM EDTA (pH 8.0), added to a 1% Megabase agarose gel (Bio-Rad, Hercules, CA) in $0.5 \times$ TBE buffer (table 2), and separated as previously described (112).

3.2.10 Ploidy Analysis

Strain ploidy was examined by flow cytometry. *C. albicans* strains were grown to log phase, and $\sim 4 \times 10^6$ cells were collected in a round-bottom microtiter plate by centrifugation. The pellets were resuspended in 20 µl of 50/50 TE (table 2); 180 µl of

95% ethanol was added dropwise, and the suspensions were stored overnight at -20°C.

The ethanol-fixed cell pellets were washed twice with 200 μ l of 50/50 TE, resuspended in 200 μ l of RNase A at 500 μ g/ml in 50/50 TE, and incubated for 2 h at 37°C. The cells were centrifuged again, and the pellets were incubated in 200 μ l of 1 mg/ml proteinase K in 50/50 TE for 2 h at 37°C. The cells were centrifuged again and washed in 50/50 TE, and the pellets were resuspended in 50 μ l of Sybr green I (1:85 dilution; Invitrogen, Carlsbad, CA) in 50/50 TE and incubated overnight in the dark. Finally, the cells were centrifuged, the pellets were resuspended in 700 μ l 50/50 TE, and ploidy was detected and analyzed on a FACScaliber flow cytometer (BD Biosciences, San Jose, CA).

4. IN VITRO EVOLUTION OF *C. albicans* DURING THE EXPOSURE TO A STEPWISE INCREASE IN FLUCONAZOLE CONCENTRATION

4.1 Introduction

The emergence of antifungal drug resistance in microbial pathogens is a process of adaptive evolution. Depending on many factors, such as population size, rate of mutation, and fitness effects, pathogen populations may be heterogeneous during the evolution in the presence of the antifungal agents, consisting of multiple beneficial mutations which compete for expansion in a process called “clonal interference” (28, 42). Determining the evolutionary dynamics during the adaption to the drugs is important for understanding the fundamental process and mechanisms underlying the emergence and spread of drug resistance at the population level.

Little information is available on the evolution of *C. albicans* in the presence of the drug, such as the frequencies with which adaptive mutants arise and expand, the evolutionary trajectories (the order of occurrence of adaptive mutations), and the potential convergence or divergence in the adaptive mechanisms between parallel populations. These are important to better understand how the pathogen evolves drug resistance in the hosts undergoing antifungal treatments. In addition, they will be useful in predicting the trajectory of a clinical infection and in rational design of appropriate therapeutic strategies.

C. albicans is an opportunistic fungal pathogen of clinical importance, causing mild superficial mucosal infections as well as life-threatening systemic infections. Rapid emergence of drug resistance was observed in the fungus both in *in vitro* and *in vivo* studies. Drug-resistant mechanisms have been extensively studied for this organism. In *C. albicans*, drug resistance can be acquired through many ways, including point mutations, loss of heterozygosity, and gross chromosomal rearrangements (109, 111, 112). However, more novel mechanisms are still required to be uncovered, as suggested by a study of a collection of drug-resistant clinical isolates (136). About 50% of the collected isolates in that study have unknown mechanisms.

Here, we monitored the *in vitro* evolution of *C. albicans* during its adaptation to a stepwise increase in fluconazole concentration, using the VERT approach which is originally designed for *S. cerevisiae* (53). Using the VERT system, the evolutionary dynamics of *C. albicans* during the *in vitro* adaptation to fluconazole was determined, which facilitated the identification and isolation of adaptive mutants. Increased drug resistance in the mutants was confirmed by fluconazole MIC measurements. Fitness effects of mutations for each adaptive isolate were evaluated both in the presence and absence of the drug by pairwise competition assays. Since aneuploidy has been associated with the drug resistance recently (109, 111), flow cytometry was used to analyze the ploidy of the adaptive mutants. In addition, the major chromosomal rearrangements have been checked by CHEF karyotype analysis for adaptive isolates

and the DNA contents present at the rearranged parts were determined by a microarray analysis.

4.2 Evolutionary Dynamics in the Presence and Absence of Fluconazole

The VERT system was modified for *C. albicans* to follow the evolutionary dynamics of two sets of parallel populations evolved either in the absence (control populations, CP-1 and CP-2) or the presence (experimental populations, DP-1 and DP-2) of fluconazole. At the start of the adaptive evolution experiments, isogenic strains differing only in the expression of different fluorescent proteins (FP) were mixed together in approximately equal numbers. Thus populations were divided into three subpopulations featuring different fluorescence and the relative proportions of each subpopulation were recorded in real time using the flow cytometry. For the drug-exposed populations, the initial drug concentration was set as 0.25µg/ml which is twice as much as the MIC of the parental strains. The concentration of fluconazole first increased to 1µg/ml at generation 64, which was followed by a further increase to 4µg/ml at generation 112. The time point at which the drug concentration changed was chosen arbitrarily. The scheme of our chemostat-based continuous culture system used to evolve the parallel populations was shown in figure 4 and the evolutionary dynamics for all four parallel populations evolved over ~170 generations were shown in figure 5.

Adaptive events occurred in subpopulations were identified by observing the expansions of those subpopulations in their relative proportions and clones that were likely to be

responsible for the observed adaptive events were isolated and named systematically according to the population from which they were isolated and the sequence in which their corresponding expansions were observed. For example, DP-1-M1 clone was the adaptive mutant isolated from the first observed expansion (M1) from the DP-1 population.

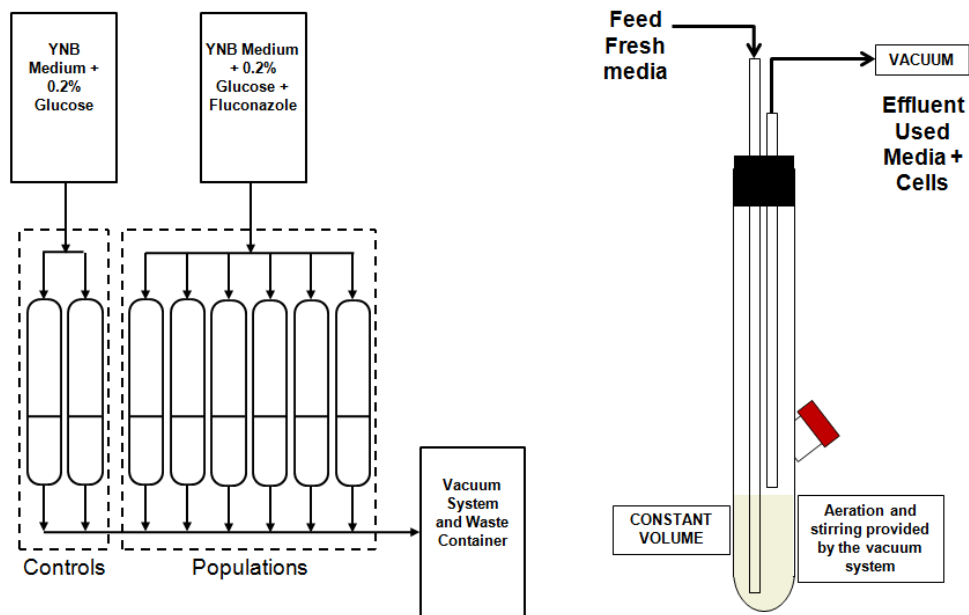


FIG. 4. Scheme of Chemostat-Based Continuous Culture System.

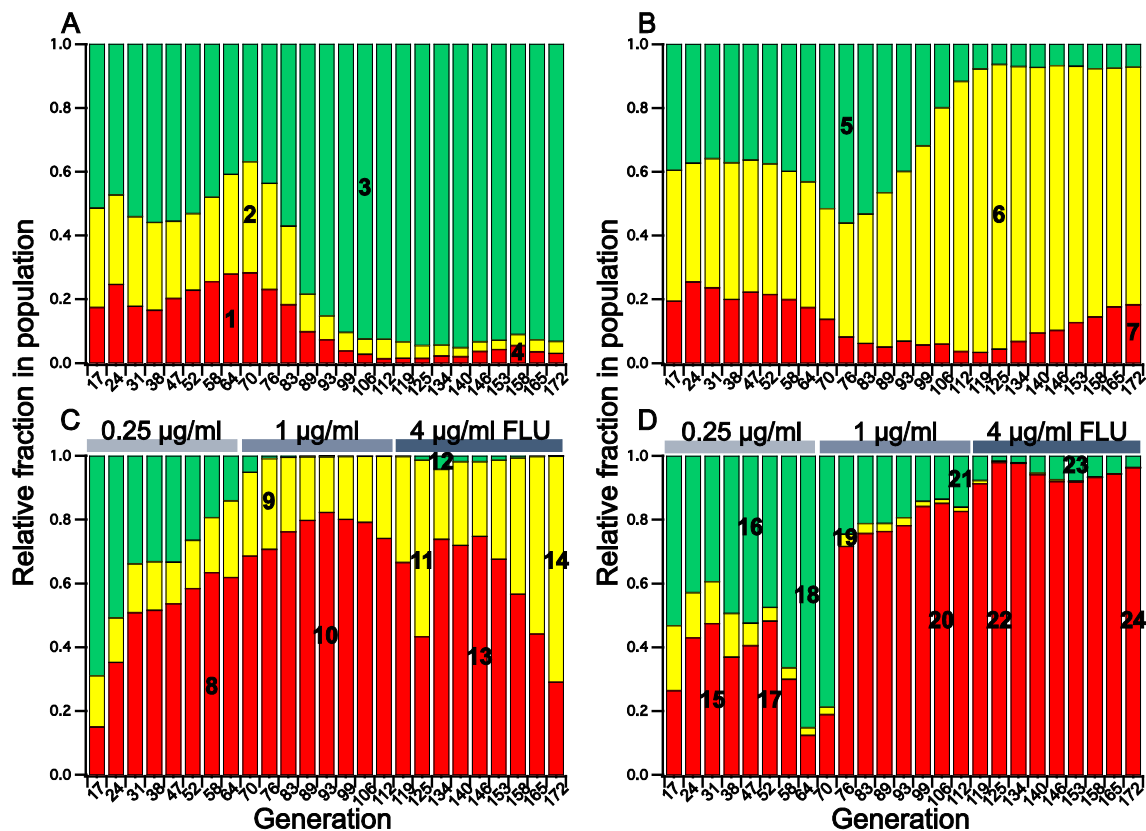


FIG. 5. Evolutionary Dynamics of Replicated *Candida albicans* Populations. Populations evolved either in the absence (CP-1 population [A] and CP-2 population [B]) or presence (DP-1 population [C] and DP-2 population [D]) of fluconazole. The colored bars represent the relative fractions of each colored subpopulation, RFP (red), GFP (green), and YFP (yellow), as determined using fluorescence-activated cell sorting (FACS). FLU, concentration of fluconazole in the feed. The subpopulations from which the adaptive mutants were isolated are numbered (taken from (Huang *et al.*, 2011))(48).

We assumed that the most competitive clone must exist in a subpopulation that is expanding. According to this assumption, subpopulations expanding just before the increase in the concentration of fluconazole will continue to expand in its relative proportion after the increase. Based on the evolutionary dynamics in the presence of drug, DP-1 population indeed follows the expected trend. The subpopulation labeled by YFP of DP-1 population started expanding at approximately generation 58 and continued its expansion following the first increase in the drug concentration at

generation 64. So does the RFP subpopulation upon the first increase in the drug concentration. The adaptive clone (DP-1-M2) isolated after the first increase in drug concentration from the YFP subpopulation indeed showed an increased fitness compared to its wild-type strain (KKCa-20) and the previous adaptive clone (DP-1-M1) in the presence of the drug (figures 6 and 7, tables 7 and 8), although no increase in the fluconazole MIC value was observed when compared to DP-1-M1 strain (0.3µg/ml). Furthermore, upon the second increase in drug concentration from 1µg/ml to 4µg/ml, at generation 112, the YFP subpopulation which started expanding at approximately generation 93 followed the expected continuous expansion as well. However, the isolated adaptive clone (DP-1-M4) from the continuously expanding subpopulation was not more competitive than the previous adaptive clone (DP-1-M3) in the presence of drug. Interestingly, the same trend was not observed in another parallel drug-exposed population (DP-2 population). After each of the stepwise increases in the fluconazole concentration, a different FP subpopulation started to expand.

Furthermore, evolutionary dynamics were compared between the *C. albicans* populations evolved in the presence and absence of fluconazole. We found an adaptive event occurred every 20 to 50 generations in the controls but more frequently, about one event per 10 to 40 generations, in the fluconazole-exposed populations. The number of adaptive events observed over ~170 generations was significantly higher in the drug-exposed populations than in those not exposed to the drug, with approximately 7 and 10 adaptive events observed in the two fluconazole-exposed populations while 3 and 4

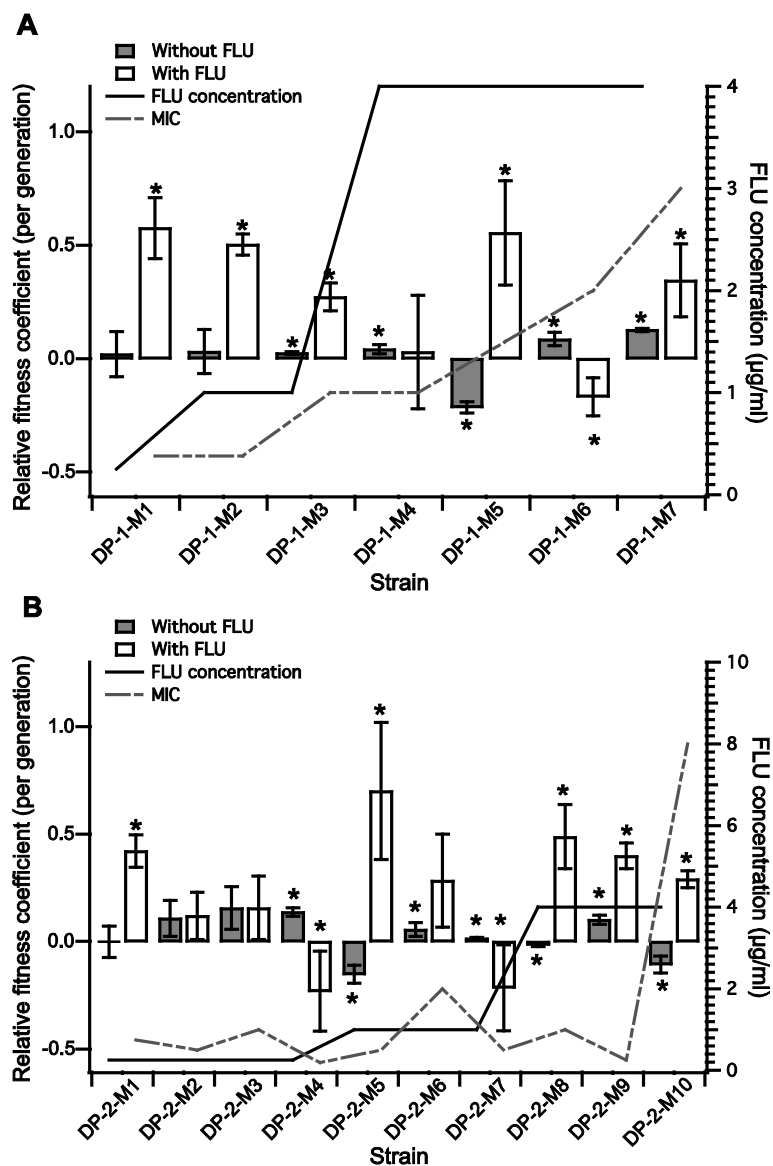


FIG. 6. Relative Fitness Coefficients of Adaptive Mutants against Previous Adaptive Mutants. The relative fitness coefficients of DP-1 (A) and DP-2 (B), in the presence of fluconazole (open bars), were measured at the drug concentration indicated by the solid line, or without fluconazole (solid bars). The data shown are the averages and standard deviations (SD) of a minimum of 3 biological replicates. The asterisks indicate that the relative fitness difference measured in the adaptive strains is statistically significant ($P \leq 0.05$, using an unpaired Student t test with unequal variance). The dashed line indicates the measured MIC of each adaptive mutant (take from (Huang *et al.*, 2011)) (48).

adaptive events observed in the control populations. Thus, the addition of the drug greatly increased the frequency of the adaptive events in the *C. albicans*.

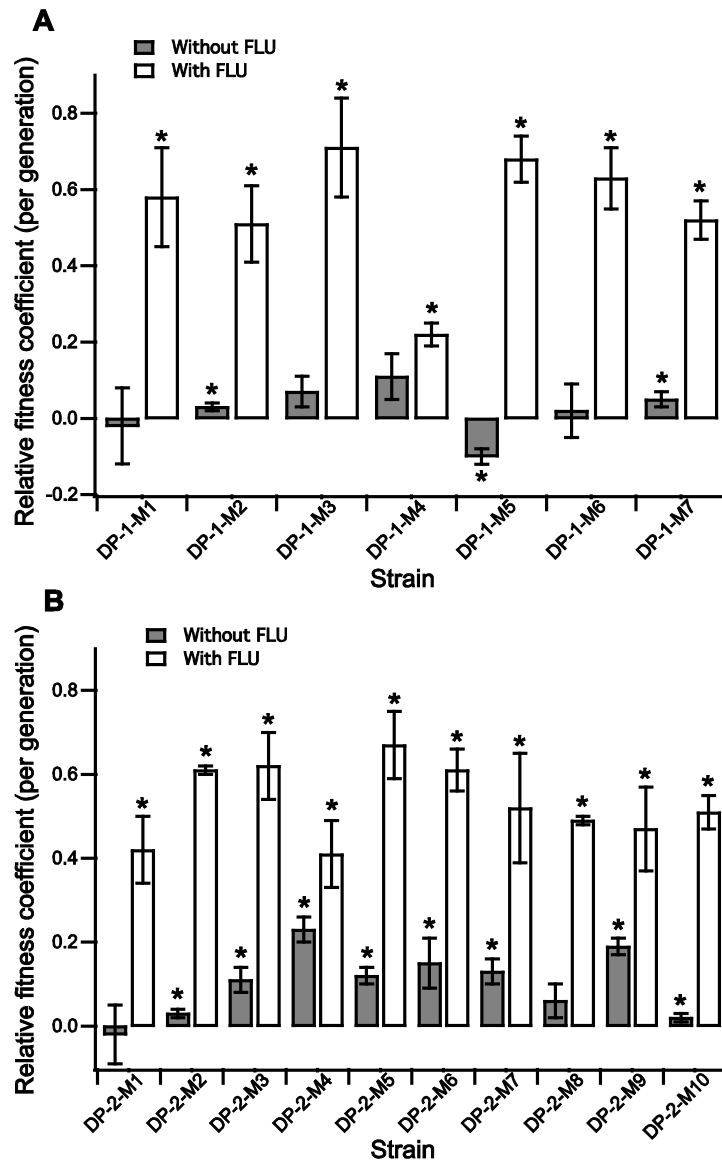


FIG. 7. Relative Fitness Coefficients of Adaptive Mutants against Parental Strains. The relative fitness coefficients of DP-1 (A) and DP-2 (B) were measured both in the presence of 0.25 $\mu\text{g/ml}$ of fluconazole (open bars) or without fluconazole (solid bars). The data shown are the averages and SD of a minimum of 3 biological replicates. The asterisks indicate that the relative fitness difference measured in the adaptive strains is statistically significant ($P < 0.05$, using an unpaired Student t test with unequal variance) (taken from (Huang *et al.*, 2011)) (48) .

TABLE 7. Adaptive Mutants Isolated from Fluconazole-Exposed and Control Populations

Adaptive event	Strain	Color ^a	FLU ($\mu\text{g/ml}$) ^b	Generations ^c	Relative fitness ^d (no drug)	p-value ^d (no drug)	Relative fitness ^d (FLU)	p-value ^d (FLU)	MIC	Floidy
parental	KKCa-14	RFP							0.125	2N
parental	KKCa-17	GFP							0.125	2N
parental	KKCa-20	YFP							0.125	2N
1	CP-1-M1	RFP	0	64	0.16 ± 0.1	0.05	NA	NA		~2N
2	CP-1-M2	YFP	0	70	0.05 ± 0.01	0.00	NA	NA		~2N
3	CP-1-M3	GFP	0	106	0.15 ± 0.02	0.00	NA	NA		~2N
4	CP-1-M4	RFP	0	158	0.24 ± 0.14	0.02	NA	NA		~2N
5	CP-2-M1	GFP	0	76	0.3 ± 0.02	0.00	NA	NA		~2N
6	CP-2-M2	YFP	0	125	0.11 ± 0.09	0.09	NA	NA		~4N
7	CP-2-M3	RFP	0	172	0.09 ± 0.11	0.12	NA	NA		~2N
8	DP-1-M1	RFP	0.25	58	0.02 ± 0.1	0.74	0.58 ± 0.13	0.01	0.38	~2N
9	DP-1-M2	YFP	1	76	0.03 ± 0.1	0.53	0.5 ± 0.05	0.00	0.38	~2N
10	DP-1-M3	RFP	1	93	0.02 ± 0.01	0.00	0.27 ± 0.06	0.01	1	~2N
11	DP-1-M4	YFP	4	125	0.04 ± 0.02	0.02	0.03 ± 0.25	0.88	1	~2N
12	DP-1-M5	GFP	4	134	-0.21 ± 0.02	0.00	0.55 ± 0.23	0.05	1.5	~2N
13	DP-1-M6	RFP	4	146	0.09 ± 0.03	0.03	-0.17 ± 0.08	0.02	2	~3N
14	DP-1-M7	YFP	4	172	0.13 ± 0.01	0.00	0.35 ± 0.16	0.06	3	~2N
15	DP-2-M1	RFP	0.25	31	0 ± 0.07	0.95	0.42 ± 0.08	0.00	0.75	~2N
16	DP-2-M2	GFP	0.25	47	0.11 ± 0.08	0.08	0.12 ± 0.11	0.20	0.5	~2N
17	DP-2-M3	RFP	0.25	52	0.16 ± 0.1	0.11	0.16 ± 0.15	0.13	1	~2N
18	DP-2-M4	GFP	0.25	64	0.14 ± 0.02	0.00	-0.32 ± 0.03	0.00	0.19	~2N
19	DP-2-M5	YFP	1	76	-0.15 ± 0.04	0.02	0.68 ± 0.34	0.03	0.5	~2N
20	DP-2-M6	RFP	1	106	0.06 ± 0.03	0.04	0.31 ± 0.20	0.12	2	~2N
21	DP-2-M7	GFP	1	112	0.02 ± 0	0.01	-0.26 ± 0.15	0.03	0.5	~2N
22	DP-2-M8	RFP	4	125	-0.02 ± 0.01	0.01	0.49 ± 0.15	0.03	1	~2N
23	DP-2-M9	GFP	4	153	0.1 ± 0.02	0.01	0.4 ± 0.06	0.00	0.25	~2N
24	DP-2-M10	RFP	4	172	-0.11 ± 0.04	0.04	0.29 ± 0.04	0.00	8	~2N

^a Fluorescent marker.

^b Fluconazole concentration in the feed.

^c Generation from which the strain was isolated.

^d Relative to previous adaptive mutant.

NA, not applicable.

TABLE 8. Relative Fitness Coefficients of Adaptive Mutants Compared to the Parental Strains

Adaptive event	Strain	Relative fitness ^a (no drug)	p-value ^a (no drug)	Relative fitness ^a (FLU)	p-value ^a (FLU)
8	DP-1-M1	-0.02 ± 0.1	0.74	0.58 ± 0.13	0.01
9	DP-1-M2	0.03 ± 0.01	0.05	0.51 ± 0.1	0.01
10	DP-1-M3	0.07 ± 0.04	0.08	0.71 ± 0.13	0.01
11	DP-1-M4	0.11 ± 0.06	0.07	0.22 ± 0.03	0.00
12	DP-1-M5	-0.1 ± 0.02	0.01	0.68 ± 0.06	0.00
13	DP-1-M6	0.02 ± 0.07	0.50	0.63 ± 0.08	0.00
14	DP-1-M7	0.05 ± 0.02	0.04	0.52 ± 0.05	0.00
15	DP-2-M1	-0.02 ± 0.07	0.63	0.42 ± 0.08	0.00
16	DP-2-M2	0.03 ± 0.01	0.00	0.61 ± 0.01	0.00
17	DP-2-M3	0.11 ± 0.03	0.03	0.62 ± 0.08	0.00
18	DP-2-M4	0.23 ± 0.03	0.01	0.41 ± 0.08	0.01
19	DP-2-M5	0.12 ± 0.02	0.01	0.67 ± 0.08	0.03
20	DP-2-M6	0.15 ± 0.06	0.05	0.61 ± 0.05	0.00
21	DP-2-M7	0.13 ± 0.03	0.01	0.52 ± 0.13	0.02
22	DP-2-M8	0.06 ± 0.04	0.08	0.49 ± 0.01	0.00
23	DP-2-M9	0.19 ± 0.02	0.00	0.47 ± 0.1	0.01
24	DP-2-M10	0.02 ± 0.01	0.03	0.51 ± 0.04	0.00

a: Relative to the parental strain of a different color.

FLU: fluconazole.

Relative fitness in units of per generation.

4.3 Fluconazole Susceptibility and Stability of the Increased Drug Resistance

Depending on the susceptibility to antifungal drugs, *C. albicans* may fail to respond to the corresponding antifungal treatments, leading to the clinical failures in treating infections caused by the pathogen. Fluconazole is the most commonly used azole drugs in treating Candidiasis due to its low toxicity to patients and good bioavailability. To determine the susceptibility of *C. albicans* to fluconazole, the isolated adaptive clones were plated on RPMI 1640 solid media and fluconazole MIC for each clone was determined by an Etest strip at 30°C. Three biological replicates were made for each clone and the highest MIC value was reported (figures 6 and 7, table 7). Interestingly,

the alteration of the MIC in the most competitive clones of the two parallel drug-exposed populations followed two different trajectories during the adaptive evolution. In DP-1 population, the trajectory of change in the MIC value is increased in a stepwise manner, which coincides with the change in the drug concentration to which the population was exposed. However, the MICs in adaptive clones of DP-2 population fluctuated during almost the entire evolution process and finally went up rapidly. Based on the MIC data, the majority of the adaptive clones isolated in this work showed higher fluconazole MIC values than their parental strains, suggesting their reduced susceptibility to the drug.

The reduced drug susceptibility observed in the adaptive clones could arise from the transient physiological response to the environmental stress or from a more stable source, genetic mutation. To figure out which mechanism is likely to contribute to the increased drug resistance in our adaptive clones, we performed stability tests, as described in Material and Methods, for all adaptive clones isolated from DP-1 and DP-2 populations. In stability tests, each clone underwent a series of transfers for about 30 generations in the absence of fluconazole and populations after the growth without the drug were subjected to fluconazole MIC measurement. The MICs of the transferred isolates and those of the original clones before the serial transfer were compared in figure 8. Importantly, the MIC values of all of the adaptive mutants did not decrease following the nonselective serial transfer and sometimes even higher than the MIC values of the parental strain, suggesting that the drug resistance is stable for a period of at least 30 generations and is likely due to the more stable genetic change.

4.4 Relative Fitness Coefficients in the Presence and Absence of Drug

Fitness effect associated with a mutation may determine the fate of the mutation in the population during the evolution. Those mutations with fitness advantage are more likely to spread and finally dominate the whole population. Thus, fitness analysis is important for understanding the fundamental principles underlying the evolutionary dynamics.

Fitness coefficients of adaptive clones responsible for observed adaptive events during the *in vitro* evolution were determined relative to two different references either by competing the adaptive isolates against the previous adaptive clone (for all isolated adaptive clones) or a parental strain with a different fluorescent marker (for isolates from drug-exposed population DP-1 and DP-2). The measurements of the relative fitness coefficients were conducted under two different conditions, both in the absence (for all adaptive clones) and presence of the drug with the same concentration as when the adaptive clones were isolated (for isolates from drug-exposed population DP-1 and DP-2). These fitness data were shown in tables 7 and 8 and figures 6 and 7.

Consistent with the reduced drug susceptibility observed in adaptive clones from drug-exposed populations by MIC measurements, adaptive isolates exhibited significant increases in fitness compared to the parental strains in the presence of the drug (figure 7 and table 8). However, when compared to the previous adaptive isolates, not all adaptive clones gained a fitness advantage in the presence of the drug (figure 6 and table 7), which is unexpected. We assumed that the most competitive clone must exist in an expanding subpopulation, thus an increase in the fitness compared to the previous

adaptive isolates in the presence of drug is supposed to be found for all adaptive clones isolated from the expanding subpopulations. This suggested that a complex interaction might exist between multiple genotypes in the evolving populations and contribute to the observed evolutionary dynamics.

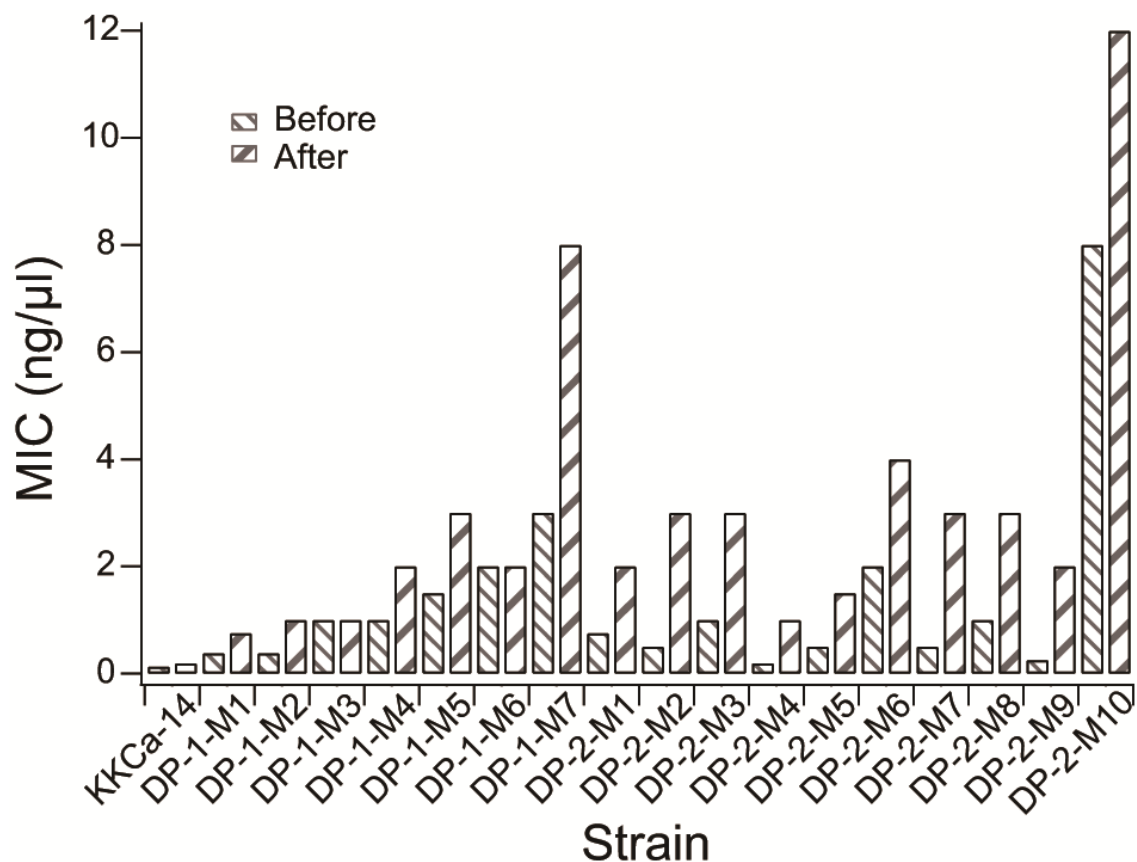


FIG. 8. Stability of Drug Resistance. Shown are the MIC values for one of the parental strains and all of the isolated adaptive mutants from the fluconazole-exposed populations before and after serial passage in the absence of the drug for more than 30 generations (Taken from (Huang *et al.*, 2011)) (48).

More importantly, for most of the adaptive clones isolated from the drug-exposed populations, the significant increases in the fitness compared to the parental strains in the

presence of drug didn't accompany with a significant fitness cost when the drug is removed. This is especially evident in adaptive clones isolated from DP-2 population and most of them exhibited fitness advantage over the parental strains both in the absence and presence of the fluconazole (figure 7 and table 8). In consistent with this observation, most of the adaptive clones with reduced drug susceptibility, as indicated by the increase in MIC value, didn't show significant decreases in the fitness compared to the parental strains in the absence of drug and instead many exhibited moderate increases in the fitness (figure 6 and table 7).

4.5 Ploidy Analysis and Chromosomal Rearrangements

Using the VERT system we successfully identified and isolated many adaptive clones and based on the stability tests stable genetic changes are most likely to be responsible for the reduced drug susceptibility and the increased fitness in the presence of the drug that observed in these adaptive isolates. The specific genetic mechanisms conferring the drug resistance as well as the fitness advantage to these isolates are of great interest, but in this work we just focused on the genetic changes in the chromosome level, including the aneuploidy and chromosomal rearrangements.

We first asked if there is any obvious sign of alterations in the ploidy of the adaptive mutants by checking the DNA content of their genomes using flow cytometry (table 7), since aneuploidy has been shown to be responsible for the drug resistance in many *C. albicans* isolates (109, 111). The majority of the adaptive isolates from the drug-

exposed populations seemed to be diploids (2N). One exception, DP-1-M6, did exist and this adaptive isolate exhibited an about 4N genome content, suggesting that it may be a tetraploid. We also performed the ploidy analysis for adaptive clones isolated from the populations evolved in the absence of the drug and similar results were obtained. All adaptive clones from these control populations except one, CP-2-M2, had an about 2N genome content, suggesting that they are also diploids. The CP-2-M2 isolate appeared to be either a triploid, since it had an about 3N genome content. Therefore, aneuploidy, an important and common resistance mechanism found elsewhere, apparently is not the major cause for the improved fitness and drug resistance of the adaptive mutants isolated in this study.

We next examined the genome of the adaptive clones from both the drug-exposed populations and the control populations by CHEF gel analysis to see if they had obvious signs of major chromosome rearrangements (figure 9). The chromosome 6 and 7 migrated differently in some adaptive mutants compared to the parental strains, which is likely due to the alterations in the lengths of the major repeat sequence tracts (66). No alterations in other larger bands were detected and no ectopic bands were observed. We further treated the genomic DNA of the adaptive clones with SfiI, a restriction enzyme which digests the genome of *C. albicans* into 2 to 3 resolvable bands per chromosome, and analyzed the migration pattern of the SfiI-treated genomic DNA. What we detected is the emergence of new bands in two adaptive clones, DP-1-M6 and DP-1-M7, isolated from independent lineages (figure 10). To determine the identity of the DNA present in

the new bands, they were excised from the gel and subject to the hybridization to microarrays of *C. albicans* open reading frame (ORFs) (31, 112). Analysis of the hybridization results showed that the new bands from the both cases correspond to a region of the chromosome R, spanning the approximate coordinates 1353 to 1907kb. The region starts at a major repeat sequence (MRS) and end at a SfiI site just past the rDNA repeats. Therefore, alterations in the lengths of the rDNA repeats are most likely to cause the formation of the new bands. In addition, we didn't detect the appearance of chromosome 5L isochromosome [i(5L)] which is associated with the increased drug resistance. In summary, we didn't detect any major gross genome rearrangements spanning the non-repetitive regions of the genome (110). According to the ploidy and chromosomal-rearrangement analysis, the improved drug resistance of the isolated adaptive clones is most likely to be explained either by the single-nucleotide polymorphisms (SNP) or other stable genome arrangements that not detected by the CHEF analysis.

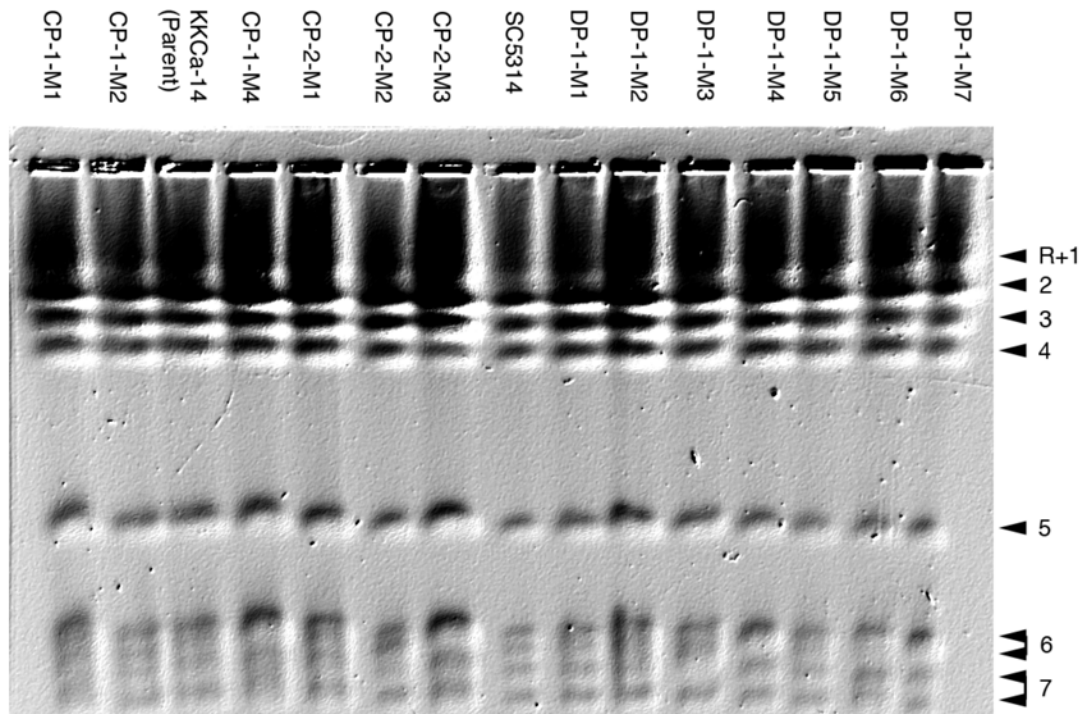


FIG. 9. Karyotype Analysis Using CHEF Gel Separation of Whole Chromosomes. Separation of whole chromosomes by CHEF gel electrophoresis and detection of chromosomal DNA by ethidium bromide staining. Identity of different chromosomes is indicated on the right. Size differences between chromosomes R and 1 are not resolved using the electrophoresis conditions used here (taken from (Huang *et al.*, 2011)) (48).

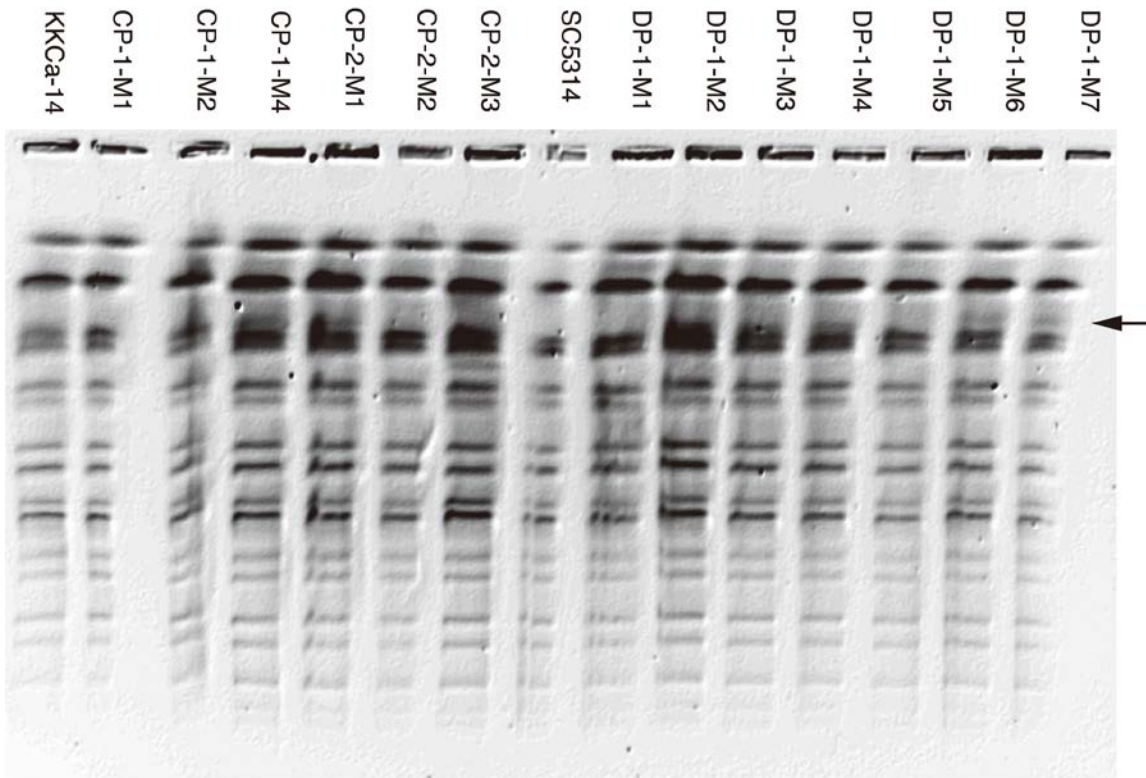


FIG. 10. Karyotype Analysis Using CHEF Gel Separation of SfiI-Digested Chromosomes. The arrow indicates a novel band migrating in strains DP-1-M6 and DP-1-M7. Subsequent analysis indicated that this band corresponds to ChrR and is hypothesized to include altered numbers of rDNA repeats (taken from (Huang *et al.*, 2011)) (48).

5. DISCUSSION

5.1 Relationship between the Evolutionary Dynamics and Relative Fitness Coefficients

Theoretically, each adaptive mutant responsible for an adaptive event observed in the evolutionary dynamics should outcompete the adaptive mutant isolated from the previous adaptive expansion in the pairwise competition experiments when the drug is present (e.g., M3 should outcompete M2 from the same population). In consistent with this, most of the adaptive isolates have a higher fitness coefficient relative to the previous adaptive isolates in the presence of fluconazole. However, some exceptions existed in the both two drug-exposed populations. When competed with the previous adaptive mutants in the presence of the drug, DP-1-M6 (generation 146), DP-2-M4 (generation 64), and DP-2-M7 (generation 112) exhibited a lower reproduction rate, suggesting they are more drug-susceptible than the previous mutants. MIC measurements of these adaptive mutants further confirmed this for DP-2-M4 and DP-2-M7 but not for DP-1-M6. When the evolutionary dynamics is checked carefully for DP-1-M5 (generation 134) and DP-1-M6, DP-1-M5 and DP-1-M6 are actually responsible for adaptive events which almost occurred at the same time (generation 125), leading the expansion of the two subpopulations against the yellow subpopulation simultaneously. Thus, DP-1-M6 is not necessarily more drug resistant than the DP-1-M5. However, DP-2-M4 and DP-2-M7 are indeed two exceptions. To account for the apparent discordance between the observations from evolutionary dynamics and pairwise competition results for the two adaptive mutants, we offered several possible explanations here. The

expansion leading to the isolation of DP-2-M4 was obviously an adaptive event based on the evolutionary dynamics, since there are three time points exhibiting the continuous increase in the green subpopulation. Based on the significant expansion rate, the proportion of the adaptive mutants DP-2-M4 in the green subpopulation is unlikely to be small. Nonetheless, among the eight colonies randomly isolated from that subpopulation at generation 134, no one exhibited increased fitness compared to the previous adaptive mutant DP-2-M3. Thus, either the actual mutant responsible for the adaptive expansion was not present in the isolates or the pairwise competition is not able to reproduce a potential beneficial interaction between DP-2-M4 and other genotypes in the evolution when DP-2-M4 was isolated. Actually, this type of beneficial interaction has been observed *in vitro* in a long-term adaptive-evolution experiment with *Escherichia coli* under glucose limitation (97), where adaptive mutants that exhibit cross-feeding of different metabolites establish a stable coexistence. Thus, we cannot rule out the possibility that complex interactions also contributed to some of the observed expansions in the populations that evolved here. In contrast, the expansion leading to the isolation of DP-2-M7 may not be an actual adaptive event and instead it is likely due to the noise in the measurements, since the relative proportions of the three FP subpopulations seem to be in a quasi-steady state from generation 99 to generation 112 and only one time point exhibited the increase in the relative proportion of the corresponding subpopulation (green) at generation 112. Alternatively, the failure of isolation of the actual adaptive mutant based on the small expansion rate and the complex interaction between multiple genotypes that not existed in pairwise competition may be the possible explanations.

5.2 Relationship between the Drug Susceptibility and Relative Fitness Coefficients

MIC Measurement has been extensively used in evaluating the drug susceptibility of fungal pathogens and the correlation of the MIC value with the *in vivo* drug resistance is well reviewed by White *et al.*(137). Although the fluconazole MIC value is currently used as a key indicator to determine whether *C. albicans* is resistant to the drug in clinical practice, evidence showed that the relationship between the MIC and the ability to adapt to an environment with drug present seems not be linear both *in vivo* and *in vitro* (24, 93). Thus, we argued that MIC alone may not predict the *in vivo* cellular response to the drug and checked our data to see if it supports this idea. The ability of the *C. albicans* isolates to adapt to the environment in our short-term evolution experiments was expressed in fitness coefficients relative to the previous isolates in the presence of the drug, determined in pairwise competition assay. The MIC values of the adaptive isolates were determined by the Etest strip assays (see materials and methods). The fitness data of the adaptive isolates was then compared to their corresponding MIC values and we found there is no correlation between the relative fitness coefficients and MIC values of the adaptive isolates. For instance, DP-1-M6 showed a significant decrease in fitness in the presence of drug relative to DP-1-M5, yet it gained an increase in the MIC value relative to DP-1-M5 (table 7). Similarly, the discordance between the MIC and the fitness can also be observed in another strain, DP-2-M9. The increase in the fitness of DP-2-M9 relative to the DP-2-M8 in the presence of the drug was accompanied with a reduction in the MIC value relative to DP-2-M8. The inconsistency between the MIC and fitness may be mostly likely due to the different growth conditions

and methods of measurement: the MIC was determined using Etest strip assays, where cells were grown on RPMI 1640 agar plates; The fitness was determined by pairwise competition experiments in chemostats with YNB broth containing fluconazole.

Therefore, our data supports the idea that the MIC alone does not reflect the fitness of a strain in the presence of the drug and thus is insufficient to predict the evolutionary outcome.

5.3 Fitness Cost of Antibiotic Resistance

Fitness effect associated with a resistant mutation plays a key role in determining the fate of the resistant genotype in the population: it will have a great impact on whether the resistant genotype can be maintained and whether the corresponding mutants will become dominant in the population. The fitness effect of a mutation is usually specific to the environment where the mutant is exposed and the effect could change among different environmental conditions. This environmental specificity of the fitness effect may be described by following four scenarios. The first scenario is antagonistic pleiotrophy (AP), which describes mutations that are beneficial in one condition but are deleterious in others. The second is mutation accumulation (MA), in which mutations that are accumulated in one environment occurred by drift are neutral in a given environment but are deleterious in another environment. The third scenario is the independent adaptation (IA), which describes mutations with beneficial roles in one environment but neutral in others. And the last scenario is cross adaptation (CA), in which mutations is beneficial in both environments. Many microbial drug-resistant

mutations have been found to fall in the first scenario, AP, with increased fitness in the presence of drug but less competitive when the drug is removed. Therefore, the proportion of the cells carrying this type mutation will decrease or the corresponding genotype may even get eliminated from the population in the absence of the drug. However, not every resistant genotype with a fitness cost will end in elimination during the evolution. Some studies showed that further evolution in the absence of the drug may generate compensatory mutations in these mutants, leading to improved fitness of these resistant genotypes (2, 67, 77, 106).

In the short-term adaptive evolution experiments reported here, we observed all scenarios described above but MA in the drug-resistant *C. albicans* isolates. An adaptive clone isolated from one population (DP-1-M5) showed a significant increase in the relative fitness compared to the parental strain in the presence of drug. The fitness of the drug resistant isolate decreased compared to the parental strain when the drug was removed (see Figure 7), demonstrating a clear case of AP. However, the majority of the isolates fall in the third or fourth categories described above, where mutations that are beneficial in the presence of the drug are either neutral or beneficial in the absence of the drug (see Figure 7). Thus, most of the adaptive clones isolated gained increased drug resistance while not became less competitive when drug is removed, which is similar to other studies of *C.albicans* (1, 24). Actually, there are some drug-resistant isolates from DP-2 population exhibiting increased fitness compared to the parental strains in the absence of the drug (see figure 7). These resistant mutations are of great interest, since

they are more likely to survive the drift and become the dominant genotype in the population.

Compared to the adaptive clones isolated from our work, Cowen *et al.* (24) observed a different picture in their study. Most isolates with increased fitness in the presence of the drug compared to the parental strain showed neutral or negative relative fitness in the absence of the drug. Interestingly, they observed the MA scenario which is absent in our study. Possible explanations to the difference between our observations and their results may be due to the differences in *C. albicans* strains used for the evolution experiments, the media used for the evolution (yeast nitrogen base versus RPMI 1640), and the evolution system (chemostat versus serial batch transfer). The use of serial batch transfer method causes a serious bottleneck effect during each transfer. Thus, it is likely that the majority of the beneficial mutations arose may get lost during the course of the serial transfers. In a continuous system, on the other hand, beneficial mutants have a higher probability of being retained in the system for further evolution.

6. CONCLUSIONS

Evolutionary dynamics of the *C. albicans* during the drug resistance development is critical for understanding the underlying fundamental principles governing the acquisition and spreading of the drug resistance within this organism in the population level. While time course samples collected from patients receiving antifungal treatments would be the ideal system to study the dynamic drug resistance development process, the limited clinical isolates and the complex host-pathogen interaction make the *in vivo* evolutionary dynamics difficult to get and reproduce. Traditional *in vitro* studies lack an efficient tool to track the appearance and expansion of the adaptive mutants in the population in real time and thus little information on the evolutionary dynamics of *C. albicans* is available. In this work, we addressed the challenges by using the VERT system to study the *in vitro* evolution of the fungus in the presence of stepwise increasing fluconazole concentration. We obtained the evolutionary dynamics for *C. albicans* evolved either in the presence or absence of the drug. In addition, we successfully identified and isolated a series of adaptive clones in a systematic manner. The Drug-resistant phenotypes are stable in these isolates for at least 30 generations in the absence of the fluconazole and most of the drug-resistant isolates didn't exhibit a fitness cost when the drug is removed. The known aneuploidy and major genome rearrangements associated with drug resistance were not detected in these adaptive clones, suggesting SNPs or other stable unknown chromosomal rearrangements may contribute to the increased drug resistance. Furthermore, we detected an increase in the

frequency of the adaptive events when the drug is present. The knowledge gained from the study may contribute to better understandings of the adaptation to the fluconazole in *C. albicans*.

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